CANADIAN JOURNAL OF RESEARCH

VOLUME 19 DECEMBER, 1941 NUMBER 12

CONTENTS

SECTION A.—PHYSICAL SCIENCES

SECTION B.—CHEMICAL SCIENCES

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

Publications and Subscriptions

The Canadian Journal of Research is issued monthly in four sections, as follows:

A. Physical Sciences
B. Chemical Sciences
C. Botanical Sciences
D. Zoological Sciences

For the present, Sections A and B are issued under a single cover, as also are Sections C and D, with separate pagination of the four sections, to permit separate binding, if desired.

Subscription rates, postage paid to any part of the world (effective 1 April, 1939), are as follows:

	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
C and D	2.50	0.50
Four sections, complete	4.00	—

The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

National Research Council, Ottawa, Canada.

Notice to Contributors

Fifty reprints of each paper are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges.

Reprinted in entirety by photo-offset.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 19, SEC. A. DECEMBER, 1941 NUMBER 12

SOME PROPERTIES OF A COMPOSITE, BIVARIATE DISTRIBUTION IN WHICH THE MEANS OF THE COMPONENT NORMAL DISTRIBUTIONS ARE LINEARLY RELATED[1]

BY F. CHARNLEY[2]

Abstract

Equations relating the statistics of a linear, composite, bivariate, normal distribution with those of the component distributions are derived for two types of data; first, for the special (ideal) case when the means represented by the samples correspond to the points of the linear continuum, and the proportions of the component populations remain constant and, second, for the practical case when the point set representing the means of the component distributions from which the samples have been drawn differs from the linear continuum, and the proportions or densities of the component populations vary along the line of relation. The results show that, if the component populations are normal and the variances and correlation coefficient in the component populations are constant, we can calculate at most only two of the parameters of the component populations from the composite data. We can, however, always calculate the values of the slope of the line of relation and the vertical variance around this line irrespective of the functional forms of the component populations, providing the means of the latter are collinear and provided also that the data can be separated into subgroups corresponding to single populations. The use of the equations is illustrated by means of a composite distribution constructed from a known component distribution.

Introduction

As is well known, the use of a regression equation to describe a relation between two variates is legitimate only if one of the variates is free from any appreciable error. This requirement is easily met in many of the problems arising in biometry, but in applications of the physical sciences to industrial problems both variates are usually subject to error and the relation required is not a regression line but the line relating the means of the two variates.

Composite distributions of this type are frequently related through time series. In the simple case when the variates are normally distributed around their respective lines of trend and the variances remain constant, the usual procedure is to fit the trends with arbitrary power series and to deduce the partial correlation between the two variates by eliminating powers of t (4). This procedure, however, is open to several serious objections. One of these is that the fitting of time series with arbitrary power series introduces a sub-

[1] *Manuscript received in original form July 9, 1941, and as revised, August 25, 1941. Contribution from the Canned Salmon Inspection Laboratory, Department of Fisheries, Vancouver, B.C.*

[2] *Chief Chemist.*

jective element that runs through all subsequent calculations and may lead to quite erroneous results. Second, the procedure involves arduous and time-consuming computations, and, third, the result gives no information regarding the nature of the relation connecting the means of the two variates.

The values of the variances and the functional forms of the two variates may, of course, be readily deduced without introducing any assumption regarding the nature of the trends, other than that the means of the two variates remain substantially constant over small intervals of time, by dividing the total interval, over which the time series extend, into a number of arbitrarily small intervals (1). This procedure, however, like the method of fitting trends, yields no information regarding the nature of the relation connecting the means of the two variates, since we can deduce this relation only from the table of corresponding values of the variates.

As far as the writer is aware, the equations connecting the parameters of the composite distribution with those of the component distributions, that are needed in deducing the relation between the means of the two variates, are not yet available in the literature, even in the simple case of linearly related normal distributions in which the respective variances and correlation coefficients remain constant. The object of this paper, therefore, was to deduce these equations for the latter simple case.

Evidently, in the composite distributions encountered in practical work, the point set representing the means of the component distributions from which the samples have been drawn will, in general, differ from the linear continuum. Furthermore, the densities or proportions of the component distributions along the line of relation will, in most instances, vary over the point set representing the individual means. For purposes of comparison, however, it will be of value first to deduce the required equations for the ideal case when the means represented by the samples correspond to the points of the linear continuum and the densities remain constant over the interval.

Assuming that we have obtained estimates of the values of the variances and the correlation coefficient r in the component populations, our principal problem is to determine the position of the line of relation, that is, to determine (i) a point on this line and (ii) the slope m of the line. In certain cases, even when we know the equation of the line of relation, we may wish to calculate one of the parameters of the component populations from the composite data. A convenient method of deducing this value is by means of the variance of the composite distribution around the line of relation.

Variance Around Line of Relation

Let AC in Fig. 1 be the series of normally correlated populations whose means are linearly related. For convenience, the individual populations may be represented by means of single probability contours (ellipses). Also, let us suppose that the populations are distributed in the interval AC, as described in the ideal case. Let B be the midpoint of AC, l the projection

of AC on the X-axis, X a deviation from the origin B, x a deviation from the mean of a single population, $P(x,y)$ the probability law of a single population, and $P(x)$, $P(y)$ the corresponding probability laws of the single variates.

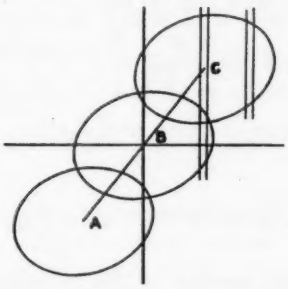


FIG. 1. *Diagrammatic representation of linear, composite, bivariate, normal distribution.*

Then the number of frequencies in the strip dX centred at B will be

$$\mu_{0B} = \int_{-1/2}^{1/2} \int_{-\infty}^{\infty} P(x,y)dydx,$$

the number of frequencies in the strip at C will be

$$\mu_{0C} = \int_{0}^{l} \int_{-\infty}^{\infty} P(x,y)dydx,$$

and the number of frequencies in a strip at X, whether the strip be within or outside the interval AC, will be

$$\mu_{0X} = \int_{X-1/2}^{X+1/2} \int_{-\infty}^{\infty} P(x,y)dydx.$$

Consider a strip at C. The vertical distance of a measure in this strip from the line of relation due to the population at C is y, and the vertical distance from the line of relation of a measure in this strip due to a population centred x units distant from C is $y - mx$. If X is the abscissa of the strip, the distance of any measure in the strip from the X-axis is therefore $y - mx + mX$.

Since the centre B is a point of symmetry, the line of best fit must clearly pass through B. Let M be the slope of any line through B (not necessarily the line of relation) and A the angle that this line makes with the X-axis. Then in a strip centred at X, the vertical distance of a measure from the line of slope M is $y - mx + (m - M)X$ and the perpendicular distance from this line is $[y - mx + (m - M)X] \cos A$.

Accordingly, in any strip at a distance X from the origin, that is, from B, the sum of the squares of the distances from the line M is

$$\Sigma\Delta_X^2 = \cos^2 A \int_{X-1/2}^{X+1/2} \int_{-\infty}^{\infty} [y - mx + (m - M)X]^2 P(x,y)dydx.$$

To integrate

$$I = \int_{-\infty}^{\infty} [y - mx + (m - M)X]^2 P(x,y)dy$$

let $k = -mx + (m - M)X$ and (2) let

$$P(x,y) = \frac{\sqrt{ac - b^2}}{2\pi} e^{-\frac{1}{2}(ax^2 + 2bxy + cy^2)},$$

where

$$\frac{ac - b^2}{c} = \frac{1}{\sigma_x^2}, \frac{b}{c} = -\frac{r\sigma_y}{\sigma_x}, \frac{ac - b^2}{a} = \frac{1}{\sigma_y^2}.$$

Then

$$I = \int_{-\infty}^{\infty} y^2 \frac{\sqrt{ac - b^2}}{2\pi} e^{-\frac{1}{2}(ax^2 + 2bxy + cy^2)} dy + \left(2k \cdot \frac{r\sigma_y}{\sigma_x} x + k^2\right) \frac{e^{-\frac{x^2}{2\sigma_x^2}}}{\sigma_x\sqrt{2\pi}}.$$

The integral in the right member of this last expression is readily obtained by writing it in the well known form

$$G = e^{-\frac{1}{2}\left(a - \frac{b^2}{c}\right)x^2} \cdot \frac{\sqrt{ac - b^2}}{2\pi} \int_{-\infty}^{\infty} y^2 e^{-\frac{1}{2}c\left(y + \frac{b}{c}x\right)^2} dy = \left[\sigma_y^2(1 - r^2) + r^2\frac{\sigma_y^2}{\sigma_x^2}x^2\right]. P(x).$$

Consequently,

$$I = \left[k^2 + 2kr\frac{\sigma_y}{\sigma_x}x + \sigma_y^2(1 - r^2) + \frac{r^2\sigma_y^2x^2}{\sigma_x^2}\right] P(x).$$

On substituting the value of k we thus obtain

$$\begin{aligned} I &= \left[\left(m^2 - 2mr\frac{\sigma_y}{\sigma_x} + r^2\frac{\sigma_y^2}{\sigma_x^2}\right)x^2 + \left(\frac{r\sigma_y}{\sigma_x} - m\right)2hXx \right. \\ &\quad \left. + h^2X^2 + \sigma_y^2(1 - r^2)\right] P(x) \\ &= [Ax^2 + BXx + CX^2 + D]\, P(x), \end{aligned}$$

where A, B, C, and D are constants determined by the values of m, σ_x, σ_y, r, and $h = m - M$. Accordingly,

$$\frac{\Sigma\Delta_X^2}{\cos^2 A} = \int_{X - l/2}^{X + l/2} (Ax^2 + BXx + CX^2 + D)P(x)dx,$$

so that on integrating and collecting terms we have

$$\begin{aligned} \frac{\Sigma\Delta_X^2}{\cos^2 A} &= -[(A + B)X + Al/2]\sigma_x^2 P(X + l/2) \\ &\quad + [(A + B)X - Al/2]\sigma_x^2 P(X - l/2) \\ &\quad + (A\sigma_x^2 + CX^2 + D)\int_{X - l/2}^{X + l/2} P(x)dx. \end{aligned}$$

Hence for all the strips, that is, on integrating with respect to X between the limits $-\infty$ and $+\infty$, we obtain

$$\frac{\Sigma\Sigma\Delta_X^2}{\cos^2 A} = Bl\sigma_x^2 + \int_{-\infty}^{\infty} (A\sigma_x^2 + CX^2 + D)\int_{X - l/2}^{X + l/2} P(x)dxdX.$$

If n is the number of populations per unit of l, the total number of measures is nl. The right member of the last equation therefore becomes

$$Bnl\sigma_x^2 + Anl\sigma_x^2 + Dnl + CnlS_X^2,$$

or, if S^2 is the perpendicular variance around the line of slope M,

$$\frac{S^2}{\cos^2 A} = (A + B)\sigma_x^2 + D + CS_X^2,$$

where S_X is the variance of the composite distribution around the Y-axis. Substituting the values of A, B, C, and D, we accordingly find

$$\frac{S^2}{\cos^2 A} = (h - m)2r\sigma_x\sigma_y + \sigma_x^2(m^2 - 2hm) + \sigma_y^2 + h^2S_X^2,$$

where $h = m - M$.

The last equation gives several interesting results. Putting $\cos^2 A = 1$ and $m = M$, that is $h = 0$, in this equation we find that the variance of the Y-variate around the line of relation is

$$S_{YL}^2 = m^2\sigma_x^2 - 2rm\sigma_x\sigma_y + \sigma_y^2.$$

Similarly, putting $\cos^2 A = 1$ and $M = 0$, we obtain the variance of the Y-variate around the origin, namely,

$$S_Y^2 = \sigma_y^2 - m^2\sigma_x^2 + m^2S_X^2.$$

But

$$nlS_X^2 = \int_{-l/2}^{l/2} n(\sigma_x^2 + X^2)dX.$$

Hence,

$$S_X^2 = \sigma_x^2 + \frac{l^2}{12}$$

and consequently,

$$S_Y^2 = \sigma_y^2 + m^2\frac{l^2}{12} = \sigma_y^2 + \frac{l'^2}{12},$$

which is the corresponding formula relating the variances of the Y-variate.

To find the slope of the line of best fit we write $\frac{\partial S^2}{\partial M} = 0$. Since $\cos^2 A = \frac{1}{1 + \tan^2 A} = \frac{1}{1 + M^2}$, the equation giving the required slope is

$$0 = \frac{1}{1+M^2}[-2r\sigma_x\sigma_y + 2m\sigma_x^2 - 2hS_X^2] - \frac{2M}{(1 + M^2)^2}[-2r\sigma_x\sigma_yM + \sigma_x^2(m^2 - 2hm) + \sigma_y^2 + h^2S_X^2].$$

From this equation and the relations $h = m - M$, $S_X^2 = \sigma_x^2 + \frac{l^2}{12}$, and $S_Y^2 = \sigma_y^2 + \frac{l'^2}{12}$, we find on collecting terms that the slope of the line of best fit is given by the equation

$$(M^2 - 1)\left(r\sigma_x\sigma_y + \frac{ll'}{12}\right) + M(S_X^2 - S_Y^2) = 0.$$

Using the result deduced in the section on the apparent correlation that, for $N = 1$, $\Sigma XY = r\sigma_x\sigma_y + \frac{ll'}{12}$, we thus see that this is merely the well

known formula for calculating the slope of the line of best fit, namely,

$$M^2 - 1 - \frac{S_Y^2 - S_X^2}{\Sigma XY} \cdot M = 0.$$

The condition that the line of relation should coincide with the line of best fit is obtained by substituting $h = 0$ in the equation resulting from putting $\frac{\partial S^2}{\partial M} = 0$. The required condition is found to be

$$-r\sigma_x\sigma_y + m^2 r\sigma_x\sigma_y + m(\sigma_x^2 - \sigma_y^2) = 0,$$

whence

$$m^2 - 1 - \frac{\sigma_y^2 - \sigma_x^2}{r\sigma_x\sigma_y} m = 0.$$

The line of relation therefore coincides with the line of best fit, if, and only if, the line of relation coincides with the line of best fit of a single population.

Finally, it should be noted that we obtain precisely similar formulae for the second variate by writing y, Y, l', m', and M' in the above equations in place of x, X, l, m, and M.

Apparent Correlation

To find the equation connecting the apparent correlation R of the composite distribution with the parameters of the component distributions we note that, in any strip at X, the vertical distance of a measure from the centre of the whole distribution is, putting $M = 0$, $y - mx + mX$. Consequently, the partial product of this strip is

$$p_{XY} = X \int_{X-l/2}^{X+l/2} \int_{-\infty}^{\infty} (y - mx + mX) P(x,y) dy dx,$$

and for all the strips we thus have

$$\Sigma p_{XY} = \int_{-\infty}^{\infty} X \int_{X-l/2}^{X+l/2} \int_{-\infty}^{\infty} (y - mx + mX) P(x,y) dy dx dX.$$

On integration this last equation gives

$$\Sigma p_{XY} = lr\sigma_x\sigma_y - lm\sigma_x^2 + \int_{-\infty}^{\infty} mX^2 \int_{X-l/2}^{X+l/2} P(x) dx dX,$$

that is,

$$\Sigma p_{XY} = lr\sigma_x\sigma_y - lm\sigma_x^2 + mNS_X^2,$$

where N is the total number of measures. The formula corresponding to this, obtained on integrating over the Y-variate, is

$$\Sigma p_{XY} = l'r\sigma_x\sigma_y - l'm'\sigma_y^2 + m'NS_Y^2,$$

in which $l' = ml$ and $m' = 1/m$.

To harmonize the two equations giving the values of Σp_{XY} we must evidently choose the units to satisfy the condition that their right members are equal. In the simple case when $m = 0$, the number of frequencies due to one com-

ponent population at a distance x from the centre of this population, which is centred at, say, X units from the origin, is

$$\int_{-\infty}^{\infty} P(x,y)dy = P(x)dX,$$

and the total number of frequencies due to this population is 1. In the derivation of the equations giving the values of Σp_{XY} we have thus assumed one component population per unit of the variate in each case.

Let n and n' be the numbers of populations per unit lengths of X and Y. The above equations then become

$$\Sigma p_{XY} = nlr\sigma_x\sigma_y - nlm\sigma_x^2 + mNS_X^2$$
$$\Sigma p_{XY} = n'l'r\sigma_x\sigma_y - n'l'm'\sigma_y^2 + m'NS_Y^2.$$

Writing the total number of measures equal to 1 we therefore find, since $N = nl = n'l'$,

$$\Sigma p_{XY} = r\sigma_x\sigma_y - m\sigma_x^2 + mS_X^2$$

and

$$\Sigma p_{XY} = r\sigma_x\sigma_y - m'\sigma_y^2 + m'S_Y^2.$$

Also, we have

$$nlS_X^2 = \int_{-l/2}^{l/2} n(\sigma_x^2 + X^2)dX,$$

so that

$$S_X^2 = \sigma_x^2 + \frac{l^2}{12}, \text{ and, similarly, } S_Y^2 = \sigma_y^2 + \frac{l'^2}{12}.$$

Remembering that $l' = ml$ and $l = m'l'$ we thus finally have

$$\Sigma p_{XY} = r\sigma_x\sigma_y + \frac{ll'}{12},$$

and

$$R = \frac{r\sigma_x\sigma_y + \frac{ll'}{12}}{\sqrt{\sigma_x^2 + \frac{l^2}{12}}\sqrt{\sigma_y^2 + \frac{l'^2}{12}}} = \frac{\Sigma XY}{NS_XS_Y}.$$

Experimental Distributions

As mentioned above, the composite distributions obtained experimentally will in general differ from the ideal case, and, in fact, will very rarely, if ever, accurately fulfil the conditions of this case. That is to say, the point set representing the means of the component distributions from which the samples have been drawn will almost always differ appreciably from the linear continuum, while, at the same time, the densities of the component distributions along the line of relation will vary from point to point. If the equations are to be of service in practical computations, they must therefore be modified so as to be rigidly applicable to the non-ideal case also.

The required equations may be readily deduced from the equations giving the moments from an arbitrary origin in terms of moments from the mean (5),

or they may be derived from the characteristic function by taking successive derivatives of the latter with respect to the variables u and v and substituting $u = 0$ and $v = 0$ in the results. Following the second method, we have for the characteristic function of a single component distribution

$$\phi(u,v) = p_1 e^{\frac{1}{2}(\sigma_x^2 u^2 + 2r\sigma_x\sigma_y uv + \sigma_y^2 v^2 + 2a_1 u + 2b_1 v)},$$

where a_1, b_1 are the means of the independent and dependent variates respectively, and p_1 is the proportion of this component in the composite distribution. Consequently,

$$(\phi)_0 = p_1,$$

$$\left(\frac{\partial\phi}{\partial u}\right)_0 = p_1 a_1,$$

$$\left(\frac{\partial^2\phi}{\partial u^2}\right)_0 = p_1\sigma_x^2 + p_1 a_1^2,$$

$$\left(\frac{\partial^2\phi}{\partial v\partial u}\right)_0 = p_1 r\sigma_x\sigma_y + p_1 a_1 b_1,$$

$$\left(\frac{\partial^3\phi}{\partial u^3}\right)_0 = 3p_1\sigma_x^2 a_1 + p_1 a_1^3,$$

$$\left(\frac{\partial^4\phi}{\partial u^4}\right)_0 = 3p_1\sigma_x^4 + 6p_1 a_1^2\sigma_x^2 + p_1 a_1^4.$$

On adjusting the origin to the means of the composite distribution and summing these equations with respect to the means of the component distributions we accordingly find

$$\mu_0 = \Sigma p_i = 1,$$
$$\mu_1 = \Sigma p_i a_i = \lambda_1 = 0;\ \mu_1' = \lambda_1' = 0,$$
$$\mu_2 = \sigma_x^2 + \Sigma p_i a_i^2 = \sigma_x^2 + \lambda_2^2;\ \mu_2' = \sigma_y^2 + m^2\lambda_2^2,$$
$$\mu_{12} = r\sigma_x\sigma_y + \Sigma p_i m a_i^2 = r\sigma_x\sigma_y + m\lambda_2^2 = r\sigma_x\sigma_y + \lambda_2\lambda_2',$$
$$\mu_3 = 3\sigma_x^2\Sigma p_i a_i + \Sigma p_i a_i^3 = 3\sigma_x^2\lambda_1 + \lambda_3^3 = \lambda_3^3;\ \mu_3' = m^3\lambda_3^3,$$
$$\mu_4 = 3\sigma_x^4 + 6\sigma_x^2\Sigma p_i a_i^2 + \Sigma p_i a_i^4 = 3\sigma_x^4 + 6\sigma_x^2\lambda_2^2 + \lambda_4^4,$$
$$\mu_4' = 3\sigma_y^4 + 6\sigma_y^2 m^2\lambda_2^2 + m^4\lambda_4^4,$$

in which the λ's and $m\lambda$'s are the moments of the means of the component distributions around the means of the composite distribution.

The required equations are now easily deduced. Multiplying the equation in μ_2 by m^2, that in μ_{12} by $2m$, and subtracting the latter from the sum of the equations giving the values of $m^2\mu_2$ and μ_2', we obtain

$$\mu_2' - 2m\mu_{12} + m^2\mu_2 = \sigma_y^2 - 2mr\sigma_x\sigma_y + m^2\sigma_x^2 = S_{YL}^2,$$

as in the ideal case. Also we have

$$\mu_2' - \sigma_y^2 = m^2\mu_2 - m^2\sigma_x^2 = m\mu_{12} - mr\sigma_x\sigma_y,$$

and the apparent correlation is

$$R = \frac{r\sigma_x\sigma_y + \lambda\lambda'}{\sqrt{\sigma_x^2 + \lambda_2^2}\sqrt{\sigma_y^2 + \lambda'^2_2}},$$

in which $\lambda' = m\lambda$.

The third moments will be zero whenever the composite distribution is symmetrical. Furthermore, since these moments will usually consist of relatively small positive or negative differences, they will not, in general, give reliable estimates of m.

Under certain conditions the value of m, however, can be calculated from the composite data if we make use of the fourth moments, for, the elimination of λ_4' from the two equations giving the values of μ_4 and μ_4' and application of the equations in μ_2 and μ_2' give the equation,

$$\mu_4' - m^4\mu_4 = 3(\mu_2')^2 - 3m^4(\mu_2)^2.$$

As might be expected, there is an analogous relation between the sixth, fourth, and second moments, namely,

$$\mu_6' - m^6\mu_6 = 15\mu_2'(\mu_4' - 2\mu_2'^2) - 15m^6\mu_2(\mu_4 - 2\mu_2^2).$$

Hence, while theoretically we can, under certain conditions, determine m from the composite data, we can calculate at most only two of the parameters of the component distributions from the composite data. In other words, in order to determine all the parameters of the composite distribution we must know the value of at least one of the quantities σ_x, σ_y, and r.

The properties of the various particular cases that arise when one or more of the parameters of the composite distribution have the value zero easily follow from the above equations. For example, when $\sigma_x = 0$, $S_{YL}^2 = \sigma_y^2$, and the line of relation becomes a linear regression line with regression coefficient m. Similarly, when the length of the interval approaches zero in both variates, the composite distribution becomes a normal distribution. The value of m is then indeterminate, since under the latter conditions $S_X^4 = 3S_X^2$, $S_X^6 = 15S_X^3$, and similar equations hold for the second variate.

The special case when $m = 0$ reveals at once a simple method of testing the hypothesis regarding the linearity of the relation between the means of the component distributions, providing it is known that the parameters σ_x, σ_y, and r remain constant over the interval and we are in possession of a means of separating the data into subgroups such that each subgroup may, with reasonable accuracy, be considered to have been drawn from a single component population. To test for linearity under these conditions we plot the means of the Y-variate against the corresponding means of the X-variate in the individual subgroups and apply the sampling limits $\pm \frac{t\sigma_y}{\sqrt{n}}$, when $m = 0$, while for $m \neq 0$ we apply the limits $b_i \pm \frac{tS_{YL}}{\sqrt{n}}$, where n is the number of measures in the subgroup.

The probabilities to be associated with the latter limits are those of a normal distribution, since the distribution of the composite population around the line of relation is normal. To demonstrate this we need only consider the moments of a single population around the line of relation. These are

$$(\mu_{YL})_n = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} (y - mx)^n P(x,y)dydx,$$

where $P(x,y)$ is the normal correlation surface in two variables. Integrating the right member of this equation for the values $n = 1, 2, 3$, etc., we find that $\mu_1 = \mu_3 = \mu_5 = 0$, $\mu_2 = \sigma_y^2 - 2mr\sigma_x\sigma_y + m^2\sigma_x^2$, $\mu_4 = 3(\sigma_y^2 - 2mr\sigma_x\sigma_y + m^2\sigma_x^2)^2$, and so on for higher moments, thus proving the statement.

Instead of plotting corresponding means we may also test the relation by comparing calculated and experimental values of one of the variances or the correlation coefficient in the component populations. The comparison of values of the correlation coefficient, however, is unsuitable for practical work, because the experimental determination of this parameter usually entails greatly increased tabulations and computations.

Lastly, we may note that the equations giving the values of m, the slope of the line of relation, and S_{YL}^2, the vertical variance around this line, are applicable to any linear, composite, bivariate distribution, so that we can always calculate the values of these two parameters irrespective of the nature of the functional forms of the component populations, provided the means of the latter are collinear.

To show that the equations

$$\frac{S_Y^2 - \sigma_y^2}{S_X^2 - \sigma_x^2} = \frac{\lambda'^2}{\lambda^2} = m^2$$

hold for any linear, composite, bivariate distribution it is necessary only to show that the relations $S_Y^2 - \sigma_y^2 = \lambda'^2$ and $S_X^2 - \sigma_x^2 = \lambda^2$ are true for any distribution of this type. Let s_p^2 be the variance, $\bar{y}_p$ the mean, and n_p the number of measures in a single population. Then the contribution of a single population to the sum of the squares of the composite distribution around the mean of the Y-variate is $n_p s_p^2 + n_p \bar{y}_p^2$. Hence, if there are k component populations and the total number of measures is N, we have

$$NS_Y^2 = \sum_{p=1}^{k} n_p s_p^2 + \sum_{p=1}^{k} n\bar{y}_p^2,$$

that is, $S_Y^2 = \sigma_y^2 + \lambda'^2$, and similarly, $S_X^2 = \sigma_x^2 + \lambda^2$. But, by hypothesis, the means are collinear. Consequently, $\bar{y}_p = m\bar{x}_p$ or $\lambda'^2 = m^2\lambda^2$, and the equations giving the value of m are true for any linear, composite, bivariate distribution.

To prove that the relation

$$S_{YL}^2 = S_Y^2 - 2mp_{XY} + m^2S_X^2$$

is true for any linear, composite, bivariate distribution we proceed in a similar manner. In a single population the vertical distance of any measure from the line of relation is $y - mx$. The contribution of a single population to the sum of the squares of the deviations of the Y-variate from the line of relation is therefore

$$\begin{aligned} n_p s_{YL}^2 &= \sum_1^{n_p} (y - mx)^2 \\ &= \sum_1^{n_p} \{Y - \bar{Y}_p - m(X - \bar{X}_p)\}^2 \end{aligned}$$

in terms of deviations from the means of the composite distribution. But $\overline{Y}_p = m\overline{X}_p$. Hence

$$n_p s^2_{YL} = \sum_1^{n_p} (Y - mX)^2 .$$

Summing with respect to the k component populations we obtain the equation giving the value of S^2_{YL}.

Numerical Example

Examples of experimental linear, composite, normal distributions will be found in other communications. In order to illustrate the simplicity of the numerical calculations, however, we shall conclude this paper by applying the preceding equations to a composite distribution that has been constructed from a known component distribution, that is, a component distribution for which we know the frequencies corresponding to given values of x and y.

In the example given below, a component distribution having the parameters $\sigma_x = \sigma_y = 1$, $r = 0.80$ was chosen, since for this value of r, four cell frequencies in each of two quadrants were immediately available in Table XXX of Pearson's "Tables for Statisticians and Biometricians" (6). The cell frequencies in the two remaining quadrants, together with those in the cells along the boundaries of the quadrants and those for values x (or y) $= 3$, were calculated by the method described by Everitt (3). In order to reduce the labour of the calculations, however, intervals of 0.5 and Simpson's Rule 1 were employed in these computations. This abridged calculation gives less accurate results than those obtained from Everitt's table, but the approximation is sufficiently close for practical purposes. For example, Everitt's table gives the value 0.1272 as the frequency in the block centred at $x = 1$, $y = 1$ on unit base, while the abridged calculation gives 0.1277. Similarly, for the block centred at $x = 2$, $y = 2$, the corresponding frequencies are 0.0260 and 0.0265 respectively. The resulting component population is shown in Table I.

Composite distributions corresponding to given values of m and given spacings may be constructed from a component population either geometrically

TABLE I

CELL FREQUENCIES IN COMPONENT NORMAL DISTRIBUTION IN WHICH $\sigma_x = \sigma_y = 1$ and $r = 0.80$

y	x							
	−3	−2	−1	0	1	2	3	Total
3					0.001	0.003	0.002	0.006
2				0.004	0.028	0.026	0.003	0.061
1			0.006	0.079	0.127	0.028	0.001	0.241
0		0.004	0.079	0.217	0.079	0.004		0.383
−1	0.001	0.028	0.127	0.079	0.006			0.241
−2	0.003	0.026	0.028	0.004				0.061
−3	0.002	0.003	0.001					0.006
Total	0.006	0.061	0.241	0.383	0.241	0.061	0.006	0.999

by summing frequencies along diagonals, or analytically, as illustrated in Table II. Table III shows a linear composite distribution derived by the latter method from the component distribution given in Table I. As will be observed from Table III, this linear composite distribution consists of three components centred on a line of slope $m = 2$.

TABLE II

DETAILS OF ANALYTIC METHOD OF CALCULATING THE FREQUENCIES IN THE ARRAY $X = 0$ OF THE COMPOSITE DISTRIBUTION CONSISTING OF THE COMPONENTS CENTRED AT (i) $X_1 = -1$, $Y_1 = -2$; (ii) $X_2 = 0$, $Y_2 = 0$; (iii) $X_3 = 1$, $Y_3 = 2$, EACH IDENTICAL WITH THE DISTRIBUTION IN TABLE I

Y	(i) $x = X + 1 = 1$, $y = Y + 2$	(ii) $x = X = 0$, $y = Y$	(iii) $x = X - 1 = -1$, $y = Y - 2$	f (From Table I)
3			1	0.006
2		2	0	0.083
1	3	1	−1	0.207
0	2	0	−2	0.273
−1	1	−1	−3	0.207
−2	0	−2		0.083
−3	−1			0.006

TABLE III

COMPOSITE DISTRIBUTION CONSISTING OF THREE COMPONENTS, $\sigma_x = \sigma_y = 1$, $r = 0.80$ CENTRED AT $X_1 = -1$, $Y_1 = -2$; $X_2 = Y_2 = 0$; $X_3 = 1$, $Y_3 = 2$, RESPECTIVELY

Y	X										
	−4	−3	−2	−1	0	1	2	3	4	f	$\bar{X}$
5							0.001	0.003	0.002	0.006	3.17
4						0.004	0.028	0.026	0.003	0.061	2.46
3					0.006	0.080	0.130	0.030	0.001	0.247	1.76
2				0.004	0.083	0.245	0.105	0.007		0.444	1.06
1			0.001	0.034	0.207	0.209	0.036	0.001		0.488	0.51
0			0.007	0.109	0.273	0.109	0.007			0.505	0.00
−1		0.001	0.036	0.209	0.207	0.034	0.001			0.488	−0.51
−2		0.007	0.105	0.245	0.083	0.004				0.444	−1.06
−3	0.001	0.030	0.130	0.080	0.006					0.247	−1.76
−4	0.003	0.026	0.028	0.004						0.061	−2.46
−5	0.002	0.003	0.001							0.006	−3.17
f	0.006	0.067	0.308	0.685	0.865	0.685	0.308	0.067	0.006		
$\bar{Y}$	−4.17	−3.34	−2.44	−1.33	0.00	1.33	2.44	3.34	4.17		

Table IV shows values of certain statistics of the distribution listed in Table III. These were calculated (i) directly from the combined distribution and (ii) from the component distributions by means of the formulae derived in the preceding sections. In computing the second series of figures the

value of $m = 2$ was employed, and Sheppard's corrections were added to the variances of the component distribution, since the measures in the latter were grouped prior to setting up the composite distribution. The results, it will be observed, agree, for the most part, to three significant figures, the limit of accuracy of the data.

TABLE IV

COMPARISON OF OBSERVED AND CALCULATED VALUES OF STATISTICS DERIVED FROM THE COMPOSITE DISTRIBUTION OF TABLE III. (i) OBSERVED; (ii) CALCULATED

$\Sigma f = 2.997$; $\Sigma fX = \Sigma fY = 0$; $\Sigma fX^2 = 5.232$; $\Sigma fY^2 = 11.226$; $\Sigma fX^4 = 25.152$; $\Sigma fY^4 = 93.930$; $\lambda^2 = 0.667$; $\lambda'^2 = 2.667$; $\Sigma f(XY)/N$, (i) 2.128, (ii) 2.133; S_x^2, (i) 1.746, (ii) 1.750; S_y^2, (i) 3.746, (ii) 3.750; S_{YL}^2, (i) 2.215, (ii) 2.217; R, (i) 0.832, (ii) 0.833; m, (i) 1.97, (ii) 2.00

References

1. CHARNLEY, F. and HARCUS, L. M. Can. J. Research, D, 18 : 410-422. 1940.
2. DARMOIS, G. Statistique mathématique. Gaston Doin et Cie, Paris. 1928.
3. EVERITT, P. F. Biometrika, 8 (3 and 4) : 385–395. 1912.
4. FISHER, R. A. Statistical methods for research workers. 5th ed. rev. and enl. Oliver and Boyd, Ltd., Edinburgh and London. 1934.
5. KELLEY, T. L. Statistical method. The Macmillan Company, New York. 1923.
6. PEARSON, K. Tables for statisticians and biometricians. 3rd ed. Part I. Cambridge University Press, London. 1930.

INDEX

CANADIAN JOURNAL OF RESEARCH

VOLUME 19
1941

SECTION A

CANADA

Published by the
NATIONAL
RESEARCH COUNCIL
of CANADA

SECTION A

INDEX TO VOLUME 19

Authors

SECTION A

INDEX TO VOLUME 19

Subjects

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 19, SEC. B | DECEMBER, 1941 | NUMBER 12

CHEMOTHERAPEUTIC STUDIES IN THE THIOPHENE SERIES

I. THE SYNTHESIS OF 2-SULPHANILAMIDO-THIOPHENE[1]

BY C. VON SEEMANN[2] AND C. C. LUCAS[3]

Abstract

A method is described for the synthesis of (1-acetylaminobenzene-4-sulphonamido)-2-thiophene and of (1-aminobenzene-4-sulphonamido)-2-thiophene, also called 2-sulphanilamido-thiophene. The acute toxicity of the above compounds has been determined and an evaluation made of their chemotherapeutic activity in experimental infections of white mice with Pneumococcus Type I; 2-sulphanilamido-thiophene shows no chemotherapeutic activity.

Introduction

In the course of a series of chemotherapeutic studies carried out in this laboratory it was thought advisable to study some thiophene derivatives. As is now well known from the fundamental work of Victor Meyer (3)*, which was extended by Steinkopf** and his collaborators, thiophene and a large number of its derivatives are closely similar to the analogous compounds of the benzene series. The reported comparative absence of toxicity of thiophene (2), which was confirmed in this laboratory, made it all the more interesting to investigate the chemotherapeutic properties of thiophene derivatives. The marked anti-bacterial activity of 2-sulphanilamido-pyridine and -thiazole suggested that the study be begun by preparing the hitherto unknown 2-sulphanilamido-thiophene.

The extreme instability of 2-aminothiophene in air presented considerable difficulty. All reactions had to be carried out in an inert atmosphere. A special apparatus was finally evolved by which the difficulties could be overcome. It allowed the reaction to be carried out in three successive steps and with complete exclusion of oxygen throughout. A yield of about 8% of 2-sulphanilamido-thiophene was obtained based upon the amount of stannic chloride salt of 2-aminothiophene hydrochloride used.

Toxicity tests carried out by Dr. P. H. Greey of the Department of Pathology and Bacteriology showed that 2-sulphanilamido-thiophene as well as its

[1] *Manuscript received August 4, 1941.*

Contribution from the Department of Medical Research, Banting Institute, University of Toronto, Toronto, Ont.

[2] *Research Assistant.*

[3] *Associate Professor.*

* *And numerous papers, mainly in "Berichte der deutschen chemischen Gesellschaft".*

** *More than 50 papers, mainly in "Liebig's Annalen".*

acetylated product had no toxic effect upon white mice in single doses up to 50 mg. Chemotherapeutic tests on white mice infected with Pneumococcus Type I showed that 2-sulphanilamido-thiophene had no curative properties in such infections. Details of the testing and further results will be published shortly by Dr. Greey.

Grateful acknowledgment is made to the Banting Research Foundation for a grant to the Department of Medical Research which supported this work.

Experimental*

I. Preparation of Thiophene

Thiophene was prepared from disodium succinate and phosphorus trisulphide according to a modification of the method of Volhard and Erdmann (7) quoted in Organic Syntheses, Vol. XII, p. 72.

II. 2-Nitrothiophene

The preparation of this substance was carried out according to the method of V. S. Babasinian (1). The purified product (yield, 88%) had a melting point of 42° C.**

III. Reduction of 2-Nitrothiophene

This procedure was carried out according to the method of Steinkopf (5) using tin filings (freshly prepared) and hydrochloric acid as the reducing agent. The resulting compound, which according to Stadler (4) has the composition: $2C_4H_5NS + 2HCl + SnCl_4$, was obtained in 78% yield.

IV. Preparation of Free 2-Aminothiophene and Condensation with 1-Acetylaminobenzene-4-Sulphonyl Chloride

A modification of the method given by Steinkopf (5) was used for the preparation of 2-aminothiophene. Owing to the great instability of 2-aminothiophene, which leads it to react with oxygen with the almost immediate formation of resinous products of polymerization, the above two steps were carried out in quick succession in the apparatus shown in Fig. 1.

The apparatus consists of two separatory funnels of 500 and 50 ml. capacity respectively (*A* and *B* in Fig. 1) and of an Erlenmeyer flask of 250 ml. (*C*). Separatory funnel *A* carries a rubber stopper with three holes through which are introduced a separatory funnel, *B*, carrying stopcock *S* 4, the gas inlet tube *G*, reaching to the bottom of funnel *A*, and the connecting tube carrying stopcock *S* 1. The connecting tube protrudes 5 mm. into the interior of *A*. The Erlenmeyer flask, *C*, carries also a rubber stopper with three holes through which the connecting tube and the two outlet tubes carrying stopcocks *S* 2 and *S* 3 extend into the interior of the flask. The connecting tube and the outlet carrying stopcock *S* 2 reach to the bottom of the Erlenmeyer flask,

* *After the present paper had been prepared for publication an article appeared (R. W. Bost and Chas. F. Starnes, J. Am. Chem. Soc. 63: 1885–1886. 1941.) describing the preparation of 2-sulphanilamido-thiophene by a slightly different method.*

** *Melting points are corrected.*

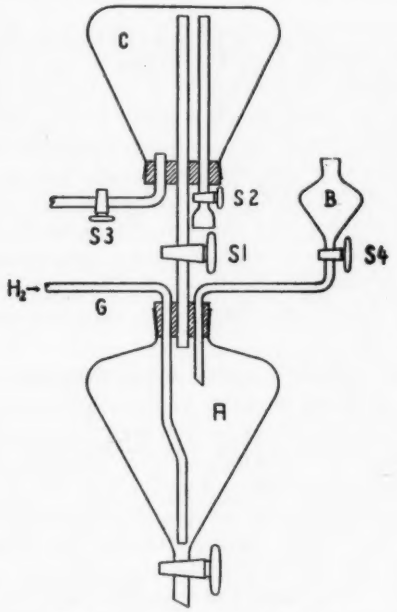


FIG. 1. *Diagram of apparatus.*

while the outlet carrying stopcock *S* 3 extends only a few millimetres into the interior. The apparatus is filled and assembled in the following way:

1-Acetylaminobenzene-4-sulphonyl chloride (4.66 gm., 0.02 mole), prepared according to Stewart's method (6), are dissolved in 50 ml. of purified acetone and the solution is introduced into the Erlenmeyer flask *C*. The stopper carrying the connecting tube and the two outlet tubes is then inserted and made secure with wire. It is essential that the three stopcocks *S* 1, *S* 2, and *S* 3 should be closed before the introduction of the stopper in order to prevent any solution from entering the tubes. The Erlenmeyer flask is then inverted and the rubber stopper on the other end of the connecting tube is closely fitted into the separatory funnel *A*, which has previously been charged with a solution of 5.4 gm. of the stannic chloride salt of thiophenine hydrochloride (equivalent to 0.02 mole of thiophenine) in 25 ml. of water and with 100 ml. of carefully purified ether. The completely assembled apparatus is then suspended on a ring stand at a suitable height and the separatory funnel *A* is submerged in an ice-bath. A two-litre beaker is especially suitable for that purpose because the reaction can be observed without interruption of the cooling. Stopcocks *S* 1 and *S* 2 are then opened while *S* 3 and *S* 4 remain closed and a vigorous current of hydrogen is introduced through the gas inlet tube, *G*. After complete elimination of the air inside the apparatus a solution of 5*N* sodium hydroxide is introduced drop by drop through the funnel *B* (with constant shaking and cooling and introduction of hydrogen)

until the precipitate initially formed has completely dissolved. The aqueous layer in separatory funnel *A* is then drawn off through the stopcock at the bottom and discarded. The ether layer containing the free thiophenine is washed with 50 ml. of water which is added in three portions through separatory funnel *B* and subsequently withdrawn at the bottom of *A* and discarded. Virtually no formation of polymerized thiophenine occurs in this process if the sodium hydroxide is added very carefully and if both the cooling and the agitation through shaking and through the rapid introduction of hydrogen are sufficient. If, however, a small amount of polymerized thiophenine should have been formed it can easily be disposed of by making it adhere to the wall of the separatory funnel *A*. Being of a very viscous nature it is readily eliminated in this way.

After having thus obtained a clear ethereal solution of thiophenine which has been freed from drops of water as thoroughly as possible, stopcock *S* 2 is closed without interrupting the current of hydrogen and the whole apparatus is rapidly and completely inverted. The Erlenmeyer flask *C* will now be at the bottom and separatory funnel *A* at the top, and as soon as stopcock *S* 3 is opened, in order to permit the escape of hydrogen, the ethereal solution of thiophenine in *A* will start running through the connecting tube into the sulphonyl chloride solution in *C* under the combined effects of gravity and hydrogen pressure. The current of hydrogen, which effects a quick mixing of the two solutions, is then interrupted for a moment, 1 ml. of pyridine is introduced into *C* through *S* 2 and the current of hydrogen is turned on again. The whole apparatus is left standing overnight at room temperature while a constant slow current of hydrogen is being maintained.

A considerable amount of a crystalline colourless substance separates. This substance is soluble in water, does not give a Beilstein reaction, and melts at 114° C. after previous slight sintering. It does not, however, contain any thiophenine, as it could be obtained from 1-acetylaminobenzene-4-sulphonyl chloride, dissolved in 1 : 1 ether-acetone with pyridine alone. The nature of this substance has so far not yet been established beyond doubt but we suppose that it is a salt-like combination of 1-acetylaminobenzene-4-sulphonic acid and pyridine. The identity of the substance obtained from the reaction mixture with that produced by mixing the sulphonyl chloride solution with pyridine in the absence of thiophenine was established by the mixed melting point which showed no depression.

The mother liquors from these crystals are then drawn off through *S* 3 into a 300 ml. flask connected with a reflux condenser by means of ground glass joints and previously swept out with hydrogen. This operation was carried out under complete exclusion of air by attaching the flask and condenser to the outlet *S* 3 by means of a Y-shaped connecting piece, using the waste hydrogen from *S* 3 to sweep out the flask, and then inclining the whole apparatus sufficiently to allow the solution to be forced by the hydrogen pressure from *C* through *S* 3 into the attached flask. The solution is then boiled under reflux and with constant introduction of hydrogen for three

hours, after which it is concentrated in a current of hydrogen to about 25 ml. The resulting dark brown solution is then dissolved in one litre of ether and transferred to a separatory funnel. The ether solution is shaken with 500 ml. of water in five portions in order to eliminate pyridine and the substance mentioned above, of which a small amount could be isolated from the water washings. The ether is then dried with sodium sulphate, filtered, and concentrated *in vacuo* to about 100 ml. After standing overnight, a considerable amount of acetylated 2-sulphanilamido-thiophene has separated and is filtered off. Further concentration of the mother liquors yields a second crop of crystals showing the same melting point as the first fraction. The substance is purified by recrystallization from acetone-ether and finally by continuous extraction with ether alone. The pure product is insoluble in water, slightly soluble in benzene and ether, very soluble in acetone and alcohol. It crystallizes from ether or acetone-ether in rhombic platelets and melts at 195° C.

The compound was recrystallized for analysis three times from acetone-ether and finally extracted with ether alone. It was dried at 60° C. and 0.1 mm. over phosphorus pentoxide, sodium hydroxide, and paraffin. Calc. for $C_{12}H_{12}O_3N_2S_2$ (mol. wt. 296.23): N, 9.46%. Found: N, 9.50, 9.44, 9.40%.

V. 2-Sulphanilamido-thiophene

Two hundred milligrams of (1-acetylaminobenzene-4-sulphonamido)-2-thiophene are dissolved in 20 ml. of 2 *N* sodium hydroxide and held in the boiling water-bath for 30 min. The clear solution is cooled with ice and carefully neutralized (litmus) with dilute hydrochloric acid. The precipitate formed crystallizes upon rubbing with a glass rod (colourless needles, which sometimes reach several millimetres in length) and is filtered after standing for some time in the refrigerator. Recrystallized from water, the substance melts at 155° C. Yield, 150 mg. = 87%. It is very soluble in alcohol and acetone, soluble in ether, insoluble in benzene. The substance is purified for analysis by dissolving in dilute sodium hydroxide, filtering, and carefully precipitating with hydrochloric acid. This process is repeated a second time and then followed by one recrystallization from water. The substance is dried at 60° and 0.1 mm. over phosphorus pentoxide and sodium hydroxide. Calc. for $C_{10}H_{10}O_2N_2S_2$ (mol. wt. = 254.22): N, 11.02%. Found: N, 11.05, 11.12%.

References

1. BABASINIAN, V. S. J. Am. Chem. Soc. 50 : 2748-2753. 1928.
2. HEFFTER, A. Arch. Physiol. 39 : 420-425. 1886.
3. MEYER, V. Die Thiophengruppe, Friedr. Vieweg und Sohn, Braunschweig. 1888.
4. STADLER, O. Ber. 18 : 1490-1492. 1885.
5. STEINKOPF, W. Ann. 403 : 17-44. 1914.
6. STEWART, J. J. Chem. Soc. 121 : 2555-2561. 1922.
7. VOLHARD, J. and ERDMANN, H. Ber. 18 : 454-455. 1885.

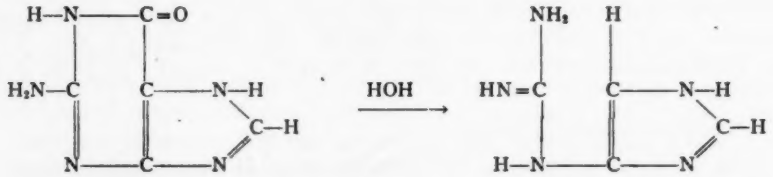
ON 4-(OR 5-) AMINOGLYOXALINE (IMINAZOLE)[1]

By G. Hunter[2] and J. A. Nelson[3]

Abstract

The dihydrochloride and sesquipicrate salts of 4-(or 5-)aminoglyoxaline have been prepared in the pure state for the first time. Previous attempts to isolate the substance have failed largely because insufficient allowance was made for the instability of the substance in aqueous media. The new glyoxaline is highly reactive and has unusual properties for a glyoxaline. It has certain properties like those of 4-(or 5-)guanidinoglyoxaline, being here proved to be a hydrolysis product of guanine.

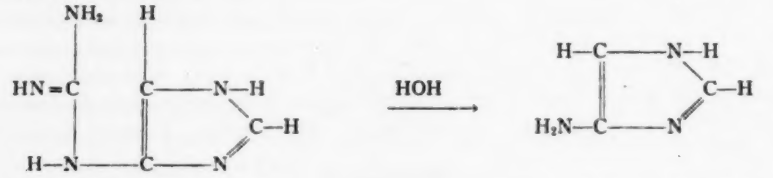
The observation of Hunter (6), subsequently confirmed by Mohr (11), that guanine on hydrolysis yields significant amounts of 4-(or 5-)guanidinoglyoxaline,



Guanine — 4-(or 5-)Guanidinoglyoxaline

in a manner closely analagous to the conversion of uric acid to allantoin, raises a number of points of both chemical and physiological interest.

It was expected on the analogy of the hydrolysis of arginine to ornithine that 4-(or 5-)guanidinoglyoxaline on further hydrolysis would yield urea and 4-(or 5-)aminoglyoxaline,

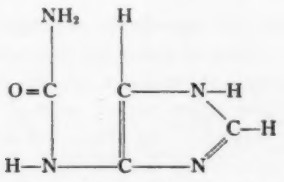


4-(or 5-)Guanidinoglyoxaline — 4-(or 5-)Aminoglyoxaline

but the actual product obtained by Hunter (7) was the intermediate 4-(or 5-) carbamidoglyoxaline.

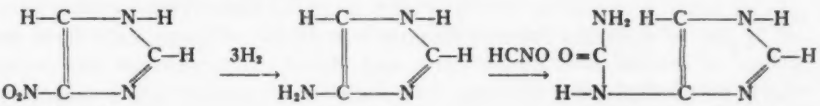
[1] *Manuscript received September 13, 1941.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton Alta.

[2] *Professor of Biochemistry.*
[3] *Research Assistant, now in Department of Chemistry, Macdonald College, McGill University, Montreal, Que.*



4-(or 5-)Carbamidoglyoxaline

However, in the products of hydrolysis of guanine, of 4-(or 5-)guanidinoglyoxaline, and of 4-(or 5-)carbamidoglyoxaline certain colour tests indicated the presence of another substance, which was probably 4-(or 5-)aminoglyoxaline. This compound has been sought by various workers, notably by Fargher and Pyman (5), Fargher (4), and Balaban (1), but it has not hitherto been isolated. Hunter and Hlynka (8) though also failing to isolate the substance were nevertheless successful in synthesizing 4-(or 5-)carbamidoglyoxaline by the addition of cyanic acid to a solution of 4-(or 5-)nitroglyoxaline reduced by sodium amalgam.



4-(or 5-)Nitroglyoxaline — 4-(or 5-)Aminoglyoxaline — 4-(or 5-)Carbamidoglyoxaline

This synthesis confirmed the existence of 4-(or 5-)aminoglyoxaline.

Because of the interest attached to this substance it was decided to make further attempts to isolate it. It was thought that it might readily be obtained from its acetyl derivative, which the writers found could be prepared by reducing 4-(or 5-)nitroglyoxaline with stannous chloride in the presence of acetic anhydride. The acetyl derivative, however, on hydrolysis with acids underwent fission of the glyoxaline nucleus, and hydrolysis with base led to similar results, though in the latter case traces of the aminoglyoxaline were detectable in the hydrolysate.

Recourse was thus made to the original line of attack. The writers were satisfied that the sodium amalgam method of reduction of the nitroglyoxaline gave good yields with minimum decomposition of the product. The solution of the problem seemed to lie in maintaining conditions during the isolation such as would avoid destruction of this very unstable glyoxaline.

As observed by Hunter and Hlynka (8), reduced solutions of the nitroglyoxaline gave a characteristic methylene-blue-like colour with the diazo reagent of Koessler and Hanke (10). The only other substance recorded as giving such a diazo test is 4-(or 5-)guanidinoglyoxaline. It might be expected that the amino- and the guanidino-substituents in the 4-(or 5-) position of the glyoxaline nucleus would have a like effect on the colour obtained in the diazo test. It was virtually certain at this stage that the diazo test

could be used to follow the synthesis or destruction of 4-(or 5-)aminoglyoxaline in solutions. Thus it was soon observed that the substance rapidly disappeared from aqueous solutions especially in the presence of acid. It was more stable in alkaline solution and seemed to hydrolyse readily in the presence of water. Accordingly, the reduction and isolation procedure was designed to maintain anhydrous conditions. The new base has been prepared as the dihydrochloride and as the sesquipicrate.

The properties of the substance are described and discussed later.

The writers failed to prepare the free base. The preparation of the salts described below is not unattended with difficulty and some uncertainty. The preparation should be carried through as expeditiously as possible.

Experimental

Synthesis of 4-(or 5-)Acetylaminoglyoxaline

4-(or 5-)Nitroglyoxaline (1 gm.), prepared according to the method of Fargher and Pyman (5), was suspended in 50 ml. of acetic anhydride and 20 ml. of glacial acetic acid and run slowly from a dropping funnel into a cold solution of 12 gm. of stannous chloride dihydrate in 40 ml. of acetic anhydride and 20 ml. of concentrated hydrochloric acid (37%). The reaction was carried out in an atmosphere of nitrogen. As the 4-(or 5-)nitroglyoxaline suspension was added, the temperature rose to about 90° C., at which temperature it was kept until the reaction was complete (usually 1.25 hr.) as judged by the diazo test.

When the reaction was complete the straw yellow solution was cooled to 0° C. and neutralized with 10 *N* sodium hydroxide, 2.5 *N* sodium hydroxide being used as the end-point was approached. The precipitated tin hydroxides were centrifuged off and washed five times with 150 ml. portions of distilled water.

The clear supernatant solution and washings were slightly acidified with sulphuric acid. To the acid solution was added just sufficient 25% aqueous mercuric acetate solution to react with all the compounds in solution. The solution was then brought to neutrality with barium hydroxide and the precipitate removed by centrifugation. After washing the mercury precipitate several times with water it was suspended in 200 ml. of 0.05 *N* sulphuric acid and treated with hydrogen sulphide. The mercuric sulphide was centrifuged off and washed with water saturated with hydrogen sulphide at least four times, using 150 ml. each time. The supernatant solution and washings were concentrated *in vacuo* to about 500 ml.

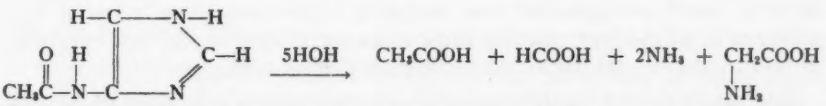
This solution was treated with 10% silver nitrate in slight excess as shown by the formation of silver oxide when a drop of the filtrate was made alkaline with barium hydroxide. The precipitate, mainly silver chloride, was centrifuged off and washed with 100 ml. portions of water four times. The supernatant and washings were made distinctly alkaline with barium hydroxide to precipitate the glyoxaline silver salt. This second precipitate was centri-

fuged off and washed with four 100 ml. portions of 0.05 *N* barium hydroxide. The silver precipitate was suspended in 150 ml. of 0.1 *N* sulphuric acid and treated with hydrogen sulphide. The silver sulphide was centrifuged off and washed four times with hydrogen sulphide water, using 100 ml. each time. The supernatant and washings were combined and concentrated to about 50 ml. *in vacuo*.

The concentrated solution was then treated with cold saturated barium hydroxide to just free the solution of sulphate ions.

The neutral solution was concentrated *in vacuo* to 10 to 15 ml. and bleached with charcoal. On further concentration of the solution to a volume of about 2 ml. the 4-(or 5-)acetylaminoglyoxaline crystallized out as white needles. This was purified, not very satisfactorily, by recrystallization from 97% dioxane. Crude yield; 50% of the theoretical. M.p. 226° C. (uncorrected). Found (micro-analysis): H, 5.70; C, 48.77; N, 34.32%. Calc. for $C_5H_7N_3O$: H, 5.64; C, 48.00; N, 33.59%.

An acetyl determination was carried out on the material using the method of Pregl for micro N-acetyl determinations. It was found that the results were twice too high for those required by the formula $C_5H_7N_3O$. This is explained from the fact that formic acid is formed through fission of the glyoxaline nucleus during hydrolysis. The presence of this acid was confirmed and it was determined quantitatively with alkaline potassium permanganate.* On making the residue from the hydrolysis alkaline with 10 *N* sodium hydroxide, ammonia was also distilled over and determined quantitatively. The final residue after the removal of the acetic acid, formic acid, and ammonia was tested for the presence of glycine by the method of Engel (3) using phenol and sodium hypochlorite. Glycine was found to be present. The following results were obtained from the hydrolytic determinations: acetic acid as CH_3CO, 33.71%; formic acid as $\equiv C-H$, 10.7%; ammonia as N_2H, 24.2%. The formula $C_5H_7N_3O$ requires: acetic acid, 34.4; formic acid, 10.4; ammonia, 24.2%. These figures give the hydrolytic products as 1 mole of acetic acid, 1 mole of formic acid, 2 moles of ammonia, and 1 mole of glycine, as expected from the following scheme (see Hunter (6)).



4-(or 5-)Acetylaminoglyoxaline Acetic Formic Glycine

* *Formic acid was determined quantitatively as follows. The distillate from the acetyl determination was made alkaline with 5 ml. of 5 N sodium hydroxide, and an excess of 0.02 N potassium permanganate was added. This solution was heated to 90° C. and kept at this temperature for 15 min. At the end of this time the solution was neutralized with sulphuric acid and 5 ml. of 10 N sulphuric acid added in excess. To this was added a slight excess of 0.02 N sodium oxalate and this was back titrated with the standard potassium permanganate. By the use of sodium oxalate in an acid solution a more sensitive end-point can be obtained.*

4-(or 5-)Acetylaminoglyoxaline readily forms a crystalline picrate in the form of needles, m.p. 208° C., and a flavianate of diamond-shaped crystals, m.p. 260° C.

The acetylaminoglyoxaline is easily soluble in water and slightly soluble in methanol. It is insoluble in ethanol, diethyl ether, acetone, and dioxane.

A minute amount of the substance gives an immediate and intense red colour with the diazo test. The nitroprusside test is entirely negative. A speck of the material on heating with ninhydrin and pyridine gives a garnet colour. Ferric chloride in acetic acid gives an orange colour. Treatment with bromine in alkali gives a brownish colour.

Preparation and Isolation of 4-(or 5-)Aminoglyoxaline Dihydrochloride

4-(or 5-)Nitroglyoxaline (1 gm.) was suspended in 150 ml. of methanol and cooled to 0° C. in an ice-bath. Nitrogen was bubbled through the solution to remove oxygen, then 3 gm. of 3% sodium amalgam was added. The suspension was then stirred vigorously to prevent the amalgam from settling to the bottom of the flask. When reduction was complete (usually 15 min.) as judged by the diazo test, the dirty bluish solution was decanted off and the remaining amalgam was washed three times with 15 ml. portions of methanol.

To the alkaline solution was added just enough mercuric acetate in methanol for complete precipitation. The precipitate was centrifuged off and washed with 200 ml. of water. This removed more of the impurities than washing with alcohol alone, and, as the mercury salt, the 4-(or 5-)aminoglyoxaline is relatively stable in water. The precipitate was washed with three 150 ml. portions of methanol after which it was assumed that all the water had been washed out. The precipitate was then suspended in methanol, and dry hydrogen chloride was bubbled in until the suspension tended to settle out rapidly. The mercury was then removed as the sulphide. During this treatment with hydrogen chloride and hydrogen sulphide the material was immersed in an ice-bath. The mercuric sulphide was centrifuged off and washed three times with 150 ml. portions of methanol. The solution was then concentrated *in vacuo* to about 20 ml. and filtered into a 3 cm. side-arm tube through a sintered glass funnel. The dark brown solution was then concentrated to 5 to 6 ml. *in vacuo* and treated with dry hydrogen chloride at 0° C. until precipitation was complete. The crystalline material was collected in a Schwinger vacuum filter and washed with several small portions of cold methanol and finally with cold pure diethyl ether.

The crude 4-(or 5-)aminoglyoxaline dihydrochloride was purified by dissolving it in a minimum volume of cold methanol and reprecipitating it with an equal volume of cold pure diethyl ether. The grey crystalline material was collected in a Schwinger vacuum filter and washed with several 0.5 ml. portions of 1 : 1 ether-methanol solution and then with pure diethyl ether. A second crop was obtained by adding an equal volume of diethyl ether to the filtrate and washings. Crystals, quadratic plates, m.p. 184° C. (uncor-

rected). Purified yield, 30% of the theoretical. Found: N, 26.83; Cl, 45.47%. Calc. for $C_3H_5N_3 . 2HCl$: N, 26.93; Cl, 45.48%. Amino nitrogen (Van Slyke):

I. 3.42 mg. substance gave 0.63 ml. of nitrogen at 25° C. and 703 mm. pressure, equals 9.72% amino N.

II. 4.34 mg. substance gave 0.74 ml. of nitrogen at 25° C. and 703 mm. pressure, equals 9.01% amino N.

The formula $C_3H_5N_3 . 2HCl$ requires 8.97% amino N.

Synthesis of 4-(or 5-)Aminoglyoxaline Sesquipicrate

4-(or 5-) Nitroglyoxaline (1 gm.) was reduced and the product precipitated with mercuric acetate as for the formation of the dihydrochloride. After washing the precipitate as above it was suspended in 150 ml. of methanol, and 3 gm. of purified picric acid was added. The glyoxaline was then freed of mercury with hydrogen sulphide and the mercuric sulphide formed was removed by centrifugation and washed with four 150 ml. portions of methanol.

The supernatant and washings were combined and evaporated *in vacuo* to 20 ml. This was transferred to a side-arm tube and evaporated further to the point at which fine needles just began to form. The solution was then filtered and washed with two 1 ml. portions of methanol. The precipitate here was discarded. The filtrate was further concentrated to about 0.5 ml. and was filtered into a sintered glass funnel. The crystals were washed with several portions of benzene to remove the excess picric acid and then with two 1 ml. portions of cold ethanol.

The material was redissolved in cold ethanol and evaporated *in vacuo* to about 0.5 ml. The crystals thus formed were collected in a sintered glass funnel and washed with two 1 ml. portions of cold ethanol. Yield of the pure picrate was 25% of the theoretical; m.p. 194° C. (uncorrected). Picric acid determinations were carried out on this material by the nitron method of Busch (2). Found: picric acid, 57.88%. Calc. for $(C_3H_5N_3)_2 . C_6H_3N_3O_7$: picric acid, 58.1%.

4-(or 5-)Aminoglyoxaline dihydrochloride and picrate are soluble in water, ethanol, and methanol. They are insoluble in benzene, acetone and diethyl ether.

Further Properties, Colour Tests, and Reactions of 4-(or 5-)Aminoglyoxaline

4-(or 5-)Aminoglyoxaline behaves as a typical glyoxaline in being precipitated by salts of mercury, silver, and by phosphotungstic acid. It gives a white precipitate with neutral ammoniacal silver solution which on warming rapidly darkens.

The base is unstable in aqueous media. A little of the dihydrochloride in water decomposes with the formation of brown and black pigments. In the presence of acids at room temperatures the glyoxaline ring suffers fission, as can readily be determined by the diazo test.

Diazo test. The substance (0.01 mg.) added to 7.5 ml. of diazo reagent gives an immediate methylene-blue–like colour, as described for guanidinoglyoxaline (6). On acidification the colour changes to a purplish-red.

Sodium nitroprusside test, as carried out by Rothera (12), is almost as sensitive as the diazo test. A blue-violet colour is obtained similar to that given by cysteine. This test, found to be given by the hydrolysates from both the guanidino- and the carbamidoglyoxaline, was thought by Hunter (7) to be more probably attributable to the presence of 4-(or 5-)hydroxyglyoxaline than to the aminoglyoxaline. The isolation of the aminoglyoxaline now settles this point and provides an explanation for other colour tests noted by Hunter (7) in the earlier work.

Ninhydrin test. When a speck of the substance is dissolved in a little water and ninhydrin and a drop of pyridine added, a grass-green colour develops slowly in the cold, and very rapidly on slight warming. The solution soon becomes brownish with a blue-green precipitate. The precipitate when separated and washed with water is soluble in alkali and reprecipitated by acid.

This test, which is quite distinct from the ninhydrin test typical of the amino acids, has previously been noted by Hunter (7) as given by the hydrolytic product of guanidino- and carbamidoglyoxalines.

Nitrous acid. As observed above, the 4-(or 5-)aminoglyoxaline yields its NH_2 nitrogen normally in the Van Slyke method. Under the Van Slyke conditions the glyoxaline ring is also broken. However when a speck of material is dissolved in a few drops of dilute acetic acid, the solution cooled in ice, and a drop of 0.5% sodium nitrite added, a faint passing green colour is observed. If a drop of this solution is quickly added to 1.1% sodium carbonate a slight greenish colour develops. If a drop is added to a mixed diazo reagent an unstable dull red colour appears; if a drop is added to 2.5 *N* sodium hydroxide a brown colour immediately appears, becoming bluish later; if a drop is added to 2.5 *N* sodium hydroxide in the presence of a little β-naphthol the colour is the same as for 2.5 *N* sodium hydroxide.

The significance of the blue colour with the nitrous acid and the red diazo test are dealt with more fully by Hunter and Hlynka (9). The fact that there is no coupling with β-naphthol indicates that the 4-(or 5-) carbon atom of the 4-(or 5-)aminoglyoxaline is not benzoid in character.

Ferric chloride in the presence of acetic acid gives a purple colour with 4-(or 5-)aminoglyoxaline.

Bromine in acid solution is absorbed by solutions of 4-(or 5-)aminoglyoxaline with fission of the glyoxaline nucleus. If, however, a speck of substance is dissolved in 2.5 *N* sodium hydroxide and a little bromine water added, a clear blue colour is produced. This is further discussed by Hunter and Hlynka (9).

Potassium permanganate. Solutions of the substance in the presence of sulphuric acid rapidly absorb potassium permanganate, with the production of a brown colour. This test is not typical of the glyoxaline nucleus. It is possibly indicative of the tautomeric change as indicated

4-(or 5-)Aminoglyoxaline 4-(or 5-)Iminoglyoxylidine

In alkaline solution, potassium permanganate gives a bright green colour with 4-(or 5-)aminoglyoxaline, 4-(or 5-)acetylaminoglyoxaline, or with histidine. This is typical of the behaviour of glyoxalines with alkaline potassium permanganate.

Cupric carbonate. When a little of the substance is heated with cupric carbonate a dirty blue solution is obtained. If the bluish precipitate is separated, washed, the copper removed as cupric sulphide, and the hydrogen sulphide removed from solution with nitrogen, a lemon-yellow solution results. On making this alkaline, a blue colour rapidly develops. This test is discussed more fully by Hunter and Hlynka (9).

The high reactivity of 4-(or 5-)aminoglyoxaline is illustrated by the following tests:

A little of the dihydrochloride is put in a dry test-tube and two drops of acetic anhydride is added. On grinding with a glass rod, fine white crystals of 4-(or 5-)acetylaminoglyoxaline appear. A diazo test will show a bright red colour as described above for this derivative.

A little of the dihydrochloride dissolved in methanol with a drop of glacial acetic acid and a little potassium cyanate soon shows evidence, when left at room temperature, of the synthesis of 4-(or 5-)carbamidoglyoxaline. After a day or so, a drop of the solution will give a clear red diazo test indicative of the conversion of the amino- to the carbamidoglyoxaline (8).

Acknowledgments

The writers are indebted to Dr. Stanley Cook of Ayerst, McKenna and Harrison, Montreal, for microanalysis.

Most of the 4- (or 5-) nitroglyoxaline used in the research was prepared by Mr. T. H. Evans.

Mr. M. M. Pechet assisted in the preparation of the 4-(or 5-) acetylaminoglyoxaline.

This work was assisted by a grant from the Associate Committee on Medical Research of the National Research Council.

References

1. BALABAN, I. E. J. Chem. Soc. 268-273. 1930.
2. BUSCH, M. Ber. 38 : 861-866. 1905.
3. ENGEL R. As quoted in J. Chem. Soc. Ab. 28 : 885. 1875.
4. FARGHER, R. G. J. Chem. Soc. 117 : 669-680. 1920.
5. FARGHER, R. G. and PYMAN, F. L. J. Chem. Soc. 115 : 217-260. 1919.
6. HUNTER, G. Biochem. J. 30 : 1183-1188. 1936.
7. HUNTER, G. Biochem. J. 30 : 1189-1201. 1936.
8. HUNTER, G. and HLYNKA, I. Biochem. J. 31 : 488-489. 1937.
9. HUNTER, G. and HLYNKA, I. Can. J. Research, B, 19(12) : 305-309. 1941.
10. KOESSLER, K. K. and HANKE, M. T. J. Biol. Chem. 39 : 497-519. 1919.
11. MOHR, M. Z. Physiol. Chem. 249 : 57-64. 1937.
12. ROTHERA, A. C. H. J. Physiol. 37 : 491-494. 1909.

ON A NEW INDIGOID FORMED FROM THE GLYOXALINE (IMINAZOLE) NUCLEUS[1]

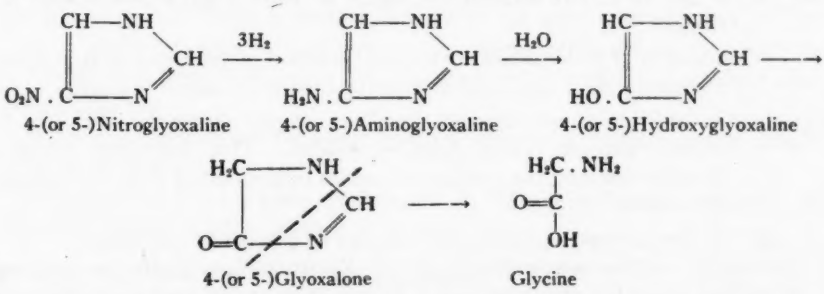
By G. Hunter[2] and I. Hlynka[3]

Abstract

Among the products of reduction of 4-(or 5-)nitroglyoxaline, formation of a blue pigment has been observed by various workers. The authors regard this pigment as a diglyoxalone containing the characteristic indigoid group, —CO . C=C . CO— . Its formation is dependent on the existence of 4-(or 5-) hydroxyglyoxaline, formed by deamination of the corresponding amino- compound. The hydroxyglyoxaline readily tautomerizes to the glyoxalone, which on oxidation condenses to form the pigment in question.

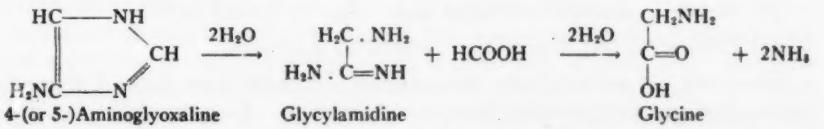
Fargher and Pyman (4) observed that when 4-(or 5-)nitroglyoxaline was reduced with alkaline sodium hyposulphite the liquors, on exposure to air, turned blue, and on acidification gave a blue precipitate. This formation of blue pigment had been previously noted by Behrend and Schmitz (1) and subsequently by others, but no one, to the writers' knowledge, has provided an explanation for its formation.

From the reduction products of 4-(or 5-)nitroglyoxaline Fargher (3) isolated glycine, and to account for it formulated the following likely chain of reactions:



assuming the glyoxalone ring to break in the manner indicated.

From the observation of Pyman (8) of amidine formation another possible mechanism of degradation of 4-(or 5-)aminoglyoxaline is:



If glycine can be formed only through the intermediary amidine, then the hypothetical hydroxyglyoxaline and glyoxalone of Fargher become open to

[1] *Manuscript received September 13, 1941.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.
[2] *Professor of Biochemistry.*
[3] *Research Assistant; now in Division of Dairy Research, Ottawa.*

question. The significance of glycine as an end-product of the degradation of certain glyoxalines has already been discussed by Hunter (5). It has been shown by Hunter and Nelson (7) to arise from 4-(or 5-)acetylaminoglyoxaline, and it can readily be shown to arise from 4-(or 5-)aminoglyoxaline by a variety of treatments, all fundamentally hydrolytic.

The above question can, however, be answered with the deaminised product of 4-(or 5-)aminoglyoxaline. This has been proved by Hunter and Nelson to deaminate quantitatively in the van Slyke machine. The product of deamination can be shown to yield glycine, so that, whether or not glycine may arise from its amidine, it can also arise through the mechanism suggested by Fargher. The probability of the existence of the hydroxyglyoxaline and glyoxalone is thus increased. The writers propose to examine other evidence for the existence of those substances, upon which is based their explanation of the blue pigment in question.

The following tests on 4-(or 5-)aminoglyoxaline dihydrochloride shed some light on the problem.

A little of the substance was treated in the cold with somewhat more than the theoretical amount of sodium nitrite in dilute acetic acid. A greenish colour appeared in the solution. Portions of it were tested as follows:

(*a*) Added to 2.5 *N* sodium hydroxide solution a dirty blue colour was formed.

(*b*) Added to 2.5 *N* sodium hydroxide solution in presence of β-naphthol a similar colour appeared, indicating absence of diazo linkage.

(*c*) On addition of one drop to the diazo reagent an immediate clear red colour, though rather faint, appeared. This indicates complete deamination and the probable presence in solution of 4-(or 5-)hydroxyglyoxaline.

(*d*) Ninhydrin test negative, thus absence of glycine at this stage.

The above solution was made about 0.1 *N* with sodium hydroxide and kept at boiling point for about 10 min. It was then cooled and made just acid with acetic acid. Portions of this were tested as follows:

(*e*) Diazo test entirely negative, indicating complete fission of all glyoxaline nuclei.

(*f*) Nessler's solution indicated mere trace of ammonia.

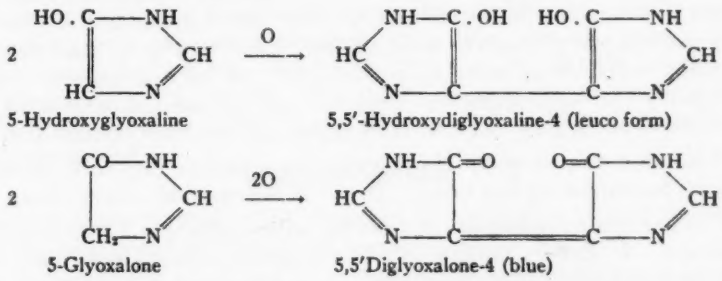
(*g*) Ninhydrin test, deep blue, indicative of glycine.

Thus the writers conclude that glycine is formed from 4-(or 5-)hydroxyglyoxaline, or more probably from the glyoxalone. From the faint red diazo test it would appear that 4-(or 5-)glyoxalone, which would not give a diazo test, is rapidly formed from 4-(or 5-)hydroxyglyoxaline. In presence of oxygen in alkaline solution blue pigment is produced.

Furthermore, Hunter (6) observed that the alkaline hydrolysates of both guanidino- and carbamidoglyoxalines when exposed to air rapidly turn blue. Hunter and Nelson have observed that the barium hydroxide hydrolysate of

4-(or 5-) acetylamino- and of 4-(or 5-)aminoglyoxaline behave similarly. So it is apparent that the blue pigment is formed from the glyoxaline nucleus, and the guanidino-, carbamido- or amino- group have no part in its production.

The work of Davidson and Baudisch (2) on urindigo, the first member of a new class of indigoids derived from pyrimidines, suggested that the blue pigment we were studying might be an indigoid derived from 4-(or 5-)glyoxalone in the following manner.



In the Experimental Section are described two preparations that the writers regard as corresponding to the leuco and blue forms of the pigment.

There may be mentioned a number of other reactions, whose mechanisms are less clear, but which probably give rise to the same blue pigment.

If a speck of 4-(or 5-)aminoglyoxaline dihydrochloride is dissolved in 2.5 *N* sodium hydroxide and a little bromine water added, a clear deep blue colour appears.

When dissolved in a little acetic acid the substance yields a purple colour with ferric chloride.

When boiled with cupric carbonate in sodium carbonate solution as described below, a blue pigment is formed.

Experimental

Preparation of 5,5′-Hydroxydiglyoxaline-4

Guanidinoglyoxaline dihydrochloride (0.5 gm.) was heated on a water-bath for seven minutes with two equivalents of freshly prepared cupric hydroxide suspended in 45 ml. of 1.1% sodium carbonate. The dark blue precipitate of copper salts that formed was separated by centrifugation and the supernatant fluid discarded. The copper salt, washed once, was suspended in water, acidified with glacial acetic acid and freed from Cu^{++} with hydrogen sulphide in the usual way. The resulting solution was freed from hydrogen sulphide by bubbling carbon dioxide rapidly through it.

The solution so obtained was lemon yellow in colour and contained about 50% of the original guanidinoglyoxaline together with the oxidation product. On addition of a drop of alkali to a test portion of the solution an immediate blue colour was produced.

The lemon yellow solution was made definitely acid to Congo red with glacial acetic acid, and an excess (about 3 ml.) of 10% silver nitrate was added. The yellow precipitate formed in acid solution was removed by centrifugation and washed with a small amount of 0.1 *N* acetic acid. It was then suspended in 5 ml. of 0.1 *N* acetic acid and Ag^+ was removed with hydrogen sulphide. The resulting solution, freed from hydrogen sulphide, was reduced to a small volume *in vacuo* and glacial acetic acid was added. It was then further evaporated to a volume of 0.5 ml. A lemon yellow precipitate was obtained by further addition of 5 ml. of glacial acetic acid. The solution and the precipitate were quickly transferred to a small test-tube, avoiding oxidation as much as possible, the test tube stoppered, and centrifuged. The supernatant was decanted and the precipitate washed twice with glacial acetic acid. The final precipitate was dried in a vacuum desiccator.

If care is not taken to avoid oxidation, the substance may turn red or blue, or may decompose, at any stage. The yield is very small, about 16 mg.

This is a crude preparation of what the writers regard as 5,5′-hydroxydiglyoxaline-4. It gives a number of colour reactions, already described. With sodium hydroxide, barium hydroxide, or ammonium hydroxide a blue colour is produced. With ferric chloride or bromine water a blue colour is also obtained. The substance is very unstable in solution but may be kept for long periods of time if dry.

Preparation of 5,5′-Diglyoxalone-4

It is possible to prepare the blue pigment, which the writers formulate as 5,5′-diglyoxalone-4, from guanidino- or carbamidoglyoxaline by the procedure just described, and by converting the leuco form to the blue pigment. The following alternative and more economical method has been used.

Nitroglyoxaline (1 gm.) suspended in 100 ml. of methanol was reduced with 50 gm. of 3% sodium amalgam in the usual manner. The alcoholic mixture was then acidified with nitric acid and the insoluble pigment was removed by centrifugation. The supernatant fluid was evaporated to dryness, the residue taken up in 25 ml. of water and a second insoluble residue was removed by centrifugation. The supernatant was made just neutral with sodium hydroxide and boiled on a water-bath for three minutes with two equivalents of cupric hydroxide in 45 ml. of 1.1% sodium carbonate. It was then centrifuged and the supernatant and washings were discarded. The precipitate was suspended in 15 ml. of water, acidified with sulphuric acid and Cu^{++} removed with hydrogen sulphide. The clear lemon yellow supernatant and washings from cupric sulphide was freed from hydrogen sulphide by bubbling through it carbon dioxide. Excess solid barium hydroxide was added. The solution acquired a deep blue colour on being shaken with air. After a half-hour it was neutralized with sulphuric acid and the barium sulphate precipitate washed until the extraneous pigment was removed. The blue pigment was then extracted from the barium sulphate precipitate with small portions of water to which a drop of 2.5 *N* sodium hydroxide was

added. The extraction was continued until all the pigment was removed. It was then precipitated by the addition of a slight excess of sulphuric acid. The pigment so obtained is contaminated with a large amount of barium sulphate, which is very difficult to remove completely. It was redissolved in water containing a minimum amount of 2.5 *N* sodium hydroxide. The solution was centrifuged at high speed to remove as much of the barium sulphate as possible, and the pigment was again reprecipitated with sulphuric acid. This procedure was repeated until the barium sulphate was removed. The pigment was finally washed with water, then alcohol, and dried *in vacuo*. The particles were too fine to be caught on a filter, and were removed by centrifugation. Yield, 7 mg. Found: N, 35.65, 34.32, 34.21%. Calc. for 5,5′-diglyoxalone-4: N, 34.15%. On the evidence of the nitrogen determination, and previous considerations as already given, it is formulated as 5-5′-diglyoxalone-4.

Acknowledgments

Acknowledgment is made to the Associate Committee on Medical Research of the National Research Council for a grant in aid of this work.

The writers would thank Mr. J. A. Nelson, for some technical assistance in the later stages of this research.

References

1. Behrend, R. and Schmitz, J. Ann. Chem. 277 : 310-339. 1893.
2. Davidson, D. and Baudisch, O. J. Biol. Chem. 64 : 619-623. 1925.
3. Fargher, R. G. J. Chem. Soc. 117 : 668-680. 1920.
4. Fargher, R. G. and Pyman, F. L. J. Chem. Soc. 115 : 217-260. 1919.
5. Hunter, G. Biochem. J. 30 : 1183-1188. 1936.
6. Hunter, G. Biochem. J. 30 : 1189-1201. 1936.
7. Hunter, G. and Nelson, J. A. Can. J. Research, B. 19(12) : 296-304. 1941.
8. Pyman, F. L. J. Chem. Soc. 121 : 2616-2626. 1922.

ON THE DETERMINATION OF URINARY GLYOXALINES (IMINAZOLES)[1]

By G. Hunter[2] and T. M. Raragosky[3]

Abstract

A method is described for the estimation of urinary glyoxalines that has certain advantages over the Koessler and Hanke method, though based on the same principles of urinary fractionation by basic lead acetate and employing the Koessler and Hanke diazo reagent. It is found that normal individuals commonly excrete 200 mg. of urinary glyoxalines, though some excrete less than half this amount, and others two or three times this amount in the course of 24 hr. Urine glyoxalines are thought to be largely of endogenous metabolic origin.

That glyoxaline derivatives are present in human urine was first proved by Engeland (4), who in 1908 isolated from it histidine and two other unidentified glyoxalines, one of which appeared to be glyoxaline amino acetic acid. Histidine has subsequently been isolated from human urine by Hunter (5), Armstrong and Walker (2), and Ackermann and Fuchs (1). Kapeller-Adler (8) has recently isolated small amounts of histamine from urines of pregnant women with eclampsia. Ackermann and Fuchs (1) had shortly before succeeded in isolating only 0.9 mg. of histamine dipicrate from 1000 litres of normal urine. It is unlikely that histamine constitutes a significant part of the glyoxalines present in urine.

In recent years interest in urinary glyoxalines has been increased by the claims notably of Kapeller-Adler (8) that histidine excretion is increased during pregnancy. The evidence of Kapeller-Adler is based largely on the finding that the urines of pregnant women give a positive Knoop test that is not found in the urine of men or of non-pregnant women. It has been suggested that there is a relationship between the excretion of histidine and the urinary gonadotropins.

The need for a method for determining urinary glyoxalines is obvious on wider considerations. Little is yet known of the metabolism of the glyoxaline nucleus, present in the essential amino-acid histidine, and in the dipeptide carnosine, found abundantly in certain mammalian muscles. On the evidence to date it is likely that much of the glyoxaline nuclear material ingested suffers fission during metabolism. Koessler and Hanke (11) and Lélu (12) have found some increase in urinary glyoxaline following high protein diets, but the increase was insufficient to account for the extra histidine intake. According to Kapeller-Adler the excretion of histidine in the urine is dependent on the presence of an enzyme, histidinase, in the liver. However, as Engeland has already indicated, histidine is not the only glyoxaline present in human urine. For a closer study of such problems the writers have elaborated the

[1] *Manuscript received September 13, 1941.*
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.
[2] *Professor of Biochemistry.*
[3] *Research Student in the Faculty of Medicine.*

present method for the determination of urinary glyoxalines. The method may be regarded as a modification of that of Koessler and Hanke (11).

Two main colour tests are available for measuring urinary glyoxalines: the Knoop test (9) and the diazo test. When bromine is added to an acid solution of histidine in very slight excess, and the solution warmed, a brown colour is produced. This is the Knoop test. It is rather highly specific for histidine, but not very sensitive, and the colour obtained is subject to variation by a variety of interfering substances. There are certain glyoxalines apparently present in urine that do not respond to the test. It will not be considered further.

The diazo test employed here depends on the fact that most glyoxalines present in biological material couple in a weakly alkaline solution with diazotized sulphanilic acid to give orange or red coloured azo products. One notable exception is anserine, in which there is a CH_3- group in the 1- position of the glyoxaline ring. The diazo test is relatively sensitive and in suitable solutions of glyoxaline gives strict proportionality between colour produced and amount of reactant present. However, when coupling takes place in an alkaline medium, the diazo test is not highly specific. Phenols, for example, couple quantitatively like glyoxalines, and numerous substances interfere with colour development. Among these may be mentioned sulphides, ammonia, purines, acetone, acetoacetic acid, and certain chromogens found in urine: see Koessler and Hanke (11) and Hunter (6). In presence of a critical concentration of an interfering substance the azo colour produced, for example, from histidine, will be yellow, or orange, instead of a clear pink, and the proportionality between histidine present and colour produced ceases to hold. In the relevant range this linear proportionality serves as the best criterion of the reliable determination of glyoxalines by azo colorimetry.

The diazo test was first applied to urine by Ehrlich (3) in 1882, as a means for detecting a substance, not yet isolated, present in typhoid and certain other febrile conditions. As it is most probably not a glyoxaline it will not be further discussed here. Ehrlich's observation, however, led to a copious literature on "the diazo reaction of urine", which has been discussed in its essentials by Hunter (6). Not till Engeland's isolation of histidine from urine, however, was it recognized that glyoxaline derivatives probably contributed largely to the diazo reaction of normal urine.

When urine is mixed with the diazo reagent the yellow or orange colour produced is never proportional to the amount of urine used, as there are always substances present that interfere with colour development. This was realized by Weiss (14) who first recognized that by treating urine with basic lead acetate, phenolic and other substances are precipitated, while the glyoxalines appear in the filtrate. The diazo reagent of Pauly (13) used by Weiss was, however, not suitable for quantitative colorimetry, as the investigations of Weiss were more concerned with the constituents of the lead precipitate than with those of the filtrate. It thus remained for Koessler and Hanke (11), with the use of their own diazo reagent (10), to elaborate a method that may

be regarded as the first with any claim to accuracy for the estimation of urinary glyoxalines.

Experimental

Preparation of Glyoxaline Fraction from Urine

Five ml. of urine is placed in a 15 ml. centrifuge tube, followed by 4.0 ml. of 40% normal lead acetate [$Pb(CH_3COO)_2 . 3H_2O$], with shaking. Four ml. of 2.0 *N* sodium hydroxide is then added and the contents thoroughly mixed. The tube is then centrifuged until the precipitate is well packed (two to five minutes). The supernatant fluid is poured off through a small filter paper, to remove floating particles of the lead precipitate, into a 50 ml. centrifuge tube. The drained lead precipitate is well suspended in 3.0 ml. of water and the tube again centrifuged. The supernatant fluid is filtered as before. The washing is repeated and the lead precipitate is discarded.

The filtrate should be as clear and colourless as water, slightly alkaline, and contain a trace of lead. The lead is precipitated by the addition of a few drops of 20% disodium hydrogen phosphate solution. The latter should be in slight excess to insure that all lead is removed at this stage.

The supernatant fluid from the centrifuged solution is decanted into a 125 ml. Erlenmeyer flask. The tube and lead phosphate precipitate are washed with a little water.

The flask is now placed on a temperature controlled hot plate at about 110° C., and the contents gently boiled to dryness. The white crystalline residue is now dissolved in a little water, transferred to a 10 ml. volumetric flask, and made up to the mark with washings. The glyoxaline fraction, now ready for colorimetry, has thus twice the volume of the native urine.

In the copious lead precipitate discarded in the method described above, there are present lead salts of such inorganic radicals as phosphate, carbonate, sulphate, and sulphide; such organic materials as sugars, acetone bodies, phenols, and purines; and such pigments as urochrome, urobilin, and their chromogens, as well as other chromogens and pigments, as bilirubin. The writers have tested urines containing glucose and giving a strong test for acetone bodies with ferric chloride, and found the lead filtrate quite free from these substances. When there is a high proportion of glucose present there is of course a larger lead precipitate.

There is no appreciable glyoxaline occluded in the lead precipitate. Koessler and Hanke (11) showed that glyoxaline acetic acid added to urine can be recovered quantitatively, and we have found that added histidine can be likewise recovered.

Evaporation to dryness of the faintly alkaline solution serves to get rid of ammonia and certain other materials potentially interfering with the subsequent determination. Glyoxalines present in human urine appear to be very stable to heat in presence of weak base. If, at the final stage, water is added and the drying repeated there is no less azo colour produced in the subsequent determination.

The method described here has several advantages over the Koessler and Hanke technique. A smaller amount of urine is used, vacuum distillation is dispensed with, and much time is saved by the use of a greater excess of lead acetate with a constant amount of standard sodium hydroxide.

Diazo Reagents

The diazo reagent according to Koessler and Hanke (10) is made from two stock solutions.

A. Nine gm. of sulphanilic acid with 90 ml. of 37% hydrochloric acid is made up to 1 litre with water.

B. Fifty gm. of 90% sodium nitrite is made up to 1 litre with water. This is best kept in a refrigerator.

The reagent is prepared by placing in a 50 ml. volumetric flask, immersed in ice-water, 1.5 ml. of solution *A* along with 1.5 ml. of solution *B*. After five minutes 6.0 ml. of solution *B* is added, and after five minutes more the flask is filled to the 50 ml. mark with cold water. The glass stopper is inserted, and the contents is thoroughly mixed. After 15 min. the reagent is ready for use. It is good for at least 24 hr. if kept cold.

A freshly prepared 1.1% solution of best quality anhydrous sodium carbonate is used to alkalinize the acid diazo reagent for each test. When thus mixed, the solution should remain perfectly clear and colourless for about 30 min.

Colour Standards

For measurement of the colour produced by the test solution, it is convenient to use an artificial comparison standard. Koessler and Hanke have shown that mixtures of Congo red and methyl orange make suitable stable standards for various glyoxalines. A standard suitable for the azo colour produced by histidine has been described by Hunter (7). It is important that the dyes be pure. This standard is suitable for the measurement of the azo colours produced by urine glyoxalines and is made as follows from two stock solutions.

Stock methyl orange. This consists of 0.1 gm. of pure methyl orange made up to 100 ml. with water.

Stock Congo red. This consists of 0.2 gm. of pure Congo red made up to 100 ml. with water.

The solutions should be kept in Pyrex glass.

Colour Standard for Histidine

About 80 ml. of distilled water is taken in a 100 ml. volumetric flask and 0.40 ml. of stock Congo red solution is added. After mixing, 0.06 ml. of stock methyl orange solution is added, the flask is filled to the mark with water, and the contents thoroughly mixed.

This colour standard is suitable for the measurement of glyoxalines in urine. Some of the above is poured into the right-hand cup of a semi-micro colorimeter of the Duboscq type.

Into the other cup is placed 2.5 ml. of 1.1% sodium carbonate solution along with 1.0 ml. of diazo reagent, and the time noted. The cup is agitated to mix. One minute later 0.5 ml. of the urinary glyoxaline fraction is added. The cup is quickly placed in the colorimeter and the contents mixed by raising and lowering the plunger several times. The plunger is then set at a depth of 10 mm. This is referred to as the *test solution*.

The colour in the test solution is at first yellow, then orange, and usually after three to four minutes, a clear pink. The standard solution plunger is moved to match. Several readings are taken between two and five minutes after mixing the test solution. Maximum readings are usually obtained at three to four minutes. Thereafter the colour gradually fades. The average of several maximum readings is recorded.

Under the above conditions, i.e., when the test solution is set at a depth of 10 mm., 0.01 mg. of histidine gives a reading of 7.4 mm. depth of standard, so that 1 mm. of standard is equivalent to 0.001352 mg. of histidine.

The volume of the test solution must be kept at 4 ml. Thus, if it is desired to use 0.1 ml. of the glyoxaline fraction above, 0.4 ml. of water should be added, before the reagents, to the colorimeter cup.

All determinations are carried out with at least two quantities of the solution under examination, and entered thus:

	Standard, mm.	Test, mm.
0.1 ml. of solution	7.1	10
0.2 ml. of solution	14.0	10

Here the proportionality is satisfactory.

The findings however might be:

0.1 ml. of solution	7.1	10
0.2 ml. of solution	11.5	10

Here the proportionality is not satisfactory, which means that the solution under test has not been adequately freed from substances that interfere with colour development. Of course the reading with the smaller quantity of glyoxaline solution will be nearer the true value than that from the greater quantity, but without linear proportionality in at least two readings, one cannot depend on the smaller quantity yielding the true reading.

From the evidence in the literature, noted above, and from our own observations (unpublished), histidine is not the only glyoxaline present in human urine. However, as histidine is probably the most constant and abundant glyoxaline present, we have expressed the urinary glyoxaline in terms of histidine.

Another consideration arises in this regard. It is well known that the azo-colours derived from different glyoxalines differ in shade and rate of colour

development. Thus the readings obtained would not always be strictly proportional, and the method might be expected to be approximate rather than precise. In practice, however, urinary glyoxalines can, in nearly all urines, be measured with a fair approach to precision. There are occasional urines from which the lead filtrates prepared as above still seem to retain substances interfering with colour development. When histidine is added to such a urine, the recovery may be greater than 100%.

Recovery of Added Histidine

To normal urine *(a)*, histidine was added in one case to the amount of 104 mg. per litre *(b)*, in the second case to the amount of 208 mg. per litre *(c)*. The three urines were treated in duplicate as described above. The detailed results are shown below.

Urine		Solution used, ml.	Reading of test, mm.	Reading of standard, mm.	Glyoxaline as histidine, mg./litre urine	Expected histidine, mg./litre urine
a	(1)	0.10	10	9.6		
		0.05	10	4.8		
	(2)	0.10	10	9.5		
		0.05	10	4.6	256.8	—
b	(1)	0.10	10	13.4		
		0.05	10	6.8		
	(2)	0.10	10	13.0		
		0.05	10	6.8	359.6	360.8
c	(1)	0.10	10	16.7		
		0.05	10	8.5		
	(2)	0.10	10	17.5		
		0.05	10	8.8	465.2	464.8

In the above the histidine values are obtained from the average of the four readings of the standard solution, and using 1 mm. of standard as equivalent to 0.001352 mg. of histidine.

The above experiment shows that histidine added to urine is fully recovered in the fractionation procedure described above.

Results

The 24-hr. urinary glyoxaline output has been determined in about one hundred normal individuals, mostly students. In Table I are the day and night excretions, in terms of histidine, for individuals where several samples were studied.

TABLE I

URINARY GLYOXALINE, AS HISTIDINE

Subjects (all men)	No. of 24-hr. samples	Average day excretion, mg.	Average night excretion, mg.	Average 24-hr. excretion, mg.
T.M.R.	17	157.8	154.8	312.6
W.L.D.	2	130.7	86.3	217.0
G.A.L.	3	369.3	344.5	713.8
J.G.R.	2	223.0	184.3	407.3

In Table II are the results from three classes of medical students. In the groups of 42 and 35 students, the urines were collected according to the directions for the Mosenthal kidney function test. In the class of 46 students, the urines were collected under ordinary conditions of diet for vitamin C determination.

TABLE II

URINARY GLYOXALINE, AS HISTIDINE, IN THREE GROUPS OF STUDENTS

No. in group (nearly all men)	Average 24-hr. excretion, mg.	Variation in 24-hr. excretion, mg./24 hr.	Average glyoxaline, mg./litre	Variation in glyoxaline, mg./litre
42	181.0	80–340	128	47–263
46	227.3	113–426	161	71–533
35	226.0	78–447	163	48–319

From Table II it may be concluded that the normal adult individual on a mixed diet commonly excretes in the urine about 200 mg. of glyoxaline, expressed as histidine, in the course of 24 hr. The daily output commonly varies from about 80 to 450 mg., although as seen from Table I (G.A.L.) more than 700 mg. may be excreted by the healthy male.

As seen from Table I, the day and night outputs of glyoxaline are not markedly different in amounts. This would suggest that urinary glyoxalines are mainly of endogenous origin.

The writers' findings suggest that the level of glyoxaline excretion is to some extent a characteristic of the individual.

Acknowledgments

The writers are indebted to Mr. J. A. Nelson for the incorporation of some of his analyses, and to Miss Sybil Fratkin for technical assistance.

This work was assisted by a grant from the Associate Committee on Medical Research of the National Research Council.

References

1. ACKERMANN, D. and FUCHS, H. G. Z. physiol. Chem. 259 : 32-34. 1939.
2. ARMSTRONG, A. R. and WALKER, E. Biochem. J. 26 : 143-146. 1932.
3. EHRLICH, P. Z. Klin. Med. 5 : 285-288. 1882.
4. ENGELAND, R. Z. physiol. Chem. 57 : 49-66. 1908.
5. HUNTER, G. Brit. Med. J. 751-752. 1922.
6. HUNTER, G. Biochem. J. 19 : 25-33. 1925.
7. HUNTER, G. Biochem. J. 19 : 42-46. 1925.
8. KAPELLER-ADLER, R. Biochem. J. 35 : 213-218. 1941.
9. KNOOP, F. Hofmeisters Beiträge. 11 : 356. 1908.
10. KOESSLER, K. K. and HANKE, M. T. J. Biol. Chem. 39 : 497-519. 1919.
11. KOESSLER, K. K. and HANKE, M. T. J. Biol. Chem. 59 : 803-834. 1924.
12. LÉLU, P. Bull Soc. Chim. Biol. 17 : 637-656. 1935.
13. PAULY, H. Z. physiol. Chem. 42 : 508-518. 1904.
14. WEISS, M. Biochem. Z. 102 : 228. 1920.

A FURTHER MODIFICATION OF THE SKRAUP SYNTHESIS OF QUINOLINE[1]

By Richard F. H. Manske[2], Frank Leger[2], and Geo. Gallagher[3]

Abstract

It has been found that recent modifications of the Skraup quinoline synthesis can be further improved as to yield and ease of operation by substituting acetylated amines for the free bases.

In the course of the preparation of a series of substituted quinolines in this laboratory, it was necessary to prepare and purify the required primary amines via their acetyl derivatives. It was considered probable that these derivatives could be used directly in the Skraup reaction, thus avoiding the necessary loss of time and material consequent upon their separate hydrolysis. Since there seems to be no previous record in which an acetylated amine has been used in the Skraup synthesis, some experiments using the cheaper acetanilide for the preparation of quinoline were carried out. The methods of Cohn (2) and of Clarke and Davis (1) (yielding 47 gm. and 41 gm. of quinoline respectively from 53.5 gm. of aniline) were used as standard procedures. In parallel experiments the aniline was replaced by an equivalent amount of acetanilide, and in either case the yield was 67 gm. Not only had the yield been substantially increased but the violence of the reaction—always serious in the Skraup synthesis—had been reduced to the point where its inception was difficult to observe. The amount of by-product tars usually obtained was also greatly reduced, presumably owing to the increased yield of quinoline.

A detailed outline of an experiment following the Cohn "Boric Acid Method" is given.

Experimental

To 20 gm. of powdered crystalline ferrous sulphate in a five litre flask, there was added in the order named, 77.6 gm. of acetanilide, 42 gm. of nitrobenzene, a solution of 35.5 gm. of boric acid in 216 gm. of glycerol, and 182 gm. of concentrated sulphuric acid, with shaking. The solution was then heated gently under a reflux condenser until it began to simmer. Mild heat was applied for one-half hour, at the end of which time the heat was increased for a further three hours.

The solution was then cooled slightly and to it was added 300 cc. of water, and the mixture was steam distilled to remove the excess nitrobenzene (about 10 gm.). The solution was then cooled and to it was added a solution of 340 gm. of sodium hydroxide in 1 litre of water. Steam distillation served

[1] *Manuscript received October 20, 1941.*

Contribution from the Division of Chemistry, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 1026.

[2] *Chemist.*

[3] *Voluntary Research Assistant.*

to remove the quinoline. The aqueous layer that separated in the distillate was further concentrated by steam distillation.

To the final combined distillate was added 70 gm. of concentrated sulphuric acid, and the resulting solution was diazotized at 8° C., with an excess of aqueous sodium nitrite. One to two grams was sufficient. The diazotized solution was heated on the steam-bath for 30 min., then steam distilled to remove volatile impurities. A solution of 100 gm. of sodium hydroxide in 400 cc. of water was added to the residual solution and the mixture was again steam distilled. The aqueous layer in the distillate was again concentrated as described above, and the quinoline was extracted from the combined distillates by means of benzene. Upon removal of the benzene and distillation of the residue under reduced pressure, there was obtained 67 gm. of water-white quinoline.

When the Clarke and Davis modification (1) was used (no boric acid) the yield of quinoline was 67 gm. When N-acetyl-*o*-anisidine was used as a starting material, with picric acid as the oxidizing agent, a good yield of 8-methoxy-quinoline was obtained. The use of 4-acetylamino-diphenyl gave a good yield of 6-phenyl-quinoline.

References

1. CLARKE, H. T., and DAVIS, ANNE W. Org. Syntheses, 2 : 79-83. 1928.
2. COHN, E. W. J. Am. Chem. Soc., 52 : 3685-3688. 1930.

CANADIAN JOURNAL OF RESEARCH

VOLUME 19
1941
SECTION B

Published by the
NATIONAL
RESEARCH COUNCIL
of CANADA

SECTION B

INDEX TO VOLUME 19

Authors

SECTION B

INDEX TO VOLUME 19

Subjects

CANADIAN JOURNAL OF RESEARCH

Notes on the Preparation of Copy

General:—Manuscripts should be typewritten, double spaced, and the original and one copy submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. All pages should be numbered.

Abstract:—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.

Illustrations:—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only. Paper ruled in green, yellow, or red should not be used. The principal co-ordinate lines should be ruled in India ink and all lines should be of sufficient thickness to reproduce well. Lettering and numerals should be of such size that they will not be less than one millimetre in height when reproduced in a cut three inches wide. If means for neat lettering are not available, lettering should be indicated in pencil only. All experimental points should be carefully drawn with instruments.

Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. Small photographs should be mounted on cardboard and those to be reproduced in groups should be so arranged and mounted. The author's name, title of paper, and figure number should be written on the back of each illustration. Captions should not be written on the illustrations, but typed on a separate page of the manuscript.

Tables:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum.

References should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper. The form of literature citation should be that used in this Journal and titles of papers should not be given. All citations should be checked with the original articles.

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.